DETECTION AND QUANTIFICATION OF ANTIBODIES TO BIOPHARMACEUTICALS

DETECTION AND QUANTIFICATION OF ANTIBODIES TO BIOPHARMACEUTICALS

Practical and Applied Considerations

EDITED BY

MICHAEL G. TOVEY

WILEY

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Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

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Library of Congress Cataloging-in-Publication Data:

Detection and quantification of antibodies to biopharmaceuticals : practical and applied considerations / [edited] by Michael G. Tovey.
p. ; cm.
Includes bibliographical references.
ISBN 978-0-470-56666-4 (cloth)
1. Immunoglobulins–Analysis. 2. Immunoassay. I. Tovey, Michael G.
[DNLM: 1. Antibodies, Neutralizing–analysis. 2. Antibodies, Neutralizing–immunology. 3. Biological Products–immunology. 4. Immunoassay–methods. QW 575]
QR186.7.D48 2011
616.07'98–dc22

2011002984

Printed in Singapore

oBook: 978-1-118-07568-5
ePDF: 978-1-118-07566-1
ePub: 978-1-118-07567-8

10 9 8 7 6 5 4 3 2 1

CONTENTS

INTRODUCTION: A PERSPECTIVE ix
Michael G. Tovey

CONTRIBUTORS xxi

PART I RISK-BASED STRATEGIES 1

1 Principles of Risk Assessment and Monitoring of Antibody Responses to Biopharmaceuticals 3
Eugen Koren, Erik Foehr, and Charles A. O'Neill

PART II REGULATORY REQUIREMENTS 13

2 Immunogenicity of Therapeutic Proteins: A Regulatory Perspective 15
Susan Kirshner

3 Guidance on Immunogenicity Assessment of Biologically Derived Therapeutic Proteins: A European Perspective 37
Meenu Wadhwa and Robin Thorpe

4 Japanese Regulatory Perspective on Immunogenicity 57
Takao Hayakawa and Akiko Ishii-Watabe

PART III PRINCIPAL TECHNOLOGIES EMPLOYED FOR THE QUANTIFICATION OF ANTI-DRUG ANTIBODIES **81**

5 Enzyme Immunoassays and Radioimmunoassays for Quantification of Anti-TNF Biopharmaceuticals and Anti-Drug Antibodies **83**
Klaus Bendtzen and Morten Svenson

6 Confirmatory Immunogenicity Assays **103**
Eric Wakshull and Daniel Coleman

7 The Use of Pharmacodynamics as a Surrogate Marker for the Detection of Anti-Drug Neutralizing Antibodies **119**
Florian Deisenhammer

8 Cell-Based Assays for the Detection of Neutralizing Antibodies to Interferon Beta (IFN-β) and Tumor Necrosis Factor Alpha (TNF-α) Inhibitors **133**
Anthony Meager

9 Detection of Neutralizing Antibodies against Interferon Beta by Real-Time RT-PCR **157**
Francesca Gilli and Antonio Bertolotto

10 Competitive Ligand-Binding Assays for the Detection of Neutralizing Antibodies **175**
Bonnie W. Wu, George R. Gunn III, and Gopi Shankar

11 The Use of Surface Plasmon Resonance for the Detection and Characterization of Antibodies **193**
Steven J. Swanson and Daniel Mytych

12 Hypersensitivity Reactions to Biopharmaceuticals: Detection and Quantification of Drug-Specific IgE Antibodies **211**
Jörgen Dahlström and Lennart Venemalm

PART IV ASSAY STANDARDIZATION AND VALIDATION **233**

13 Standardization and Validation of Immunoassays **235**
Daniel Kramer

14 Standardization and Validation of Cell-Based Assays for the Detection of Neutralizing Anti-Drug Antibodies **243**
Deborah Finco-Kent and Amy Grenham

15 Standardization of Neutralizing Antibody Unitage by Bioassay Design: Constant Antigen and Constant Antibody Methodology **269**
Sidney E. Grossberg, Yoshimi Kawade, and Leslie D. Grossberg

PART V STATISTICAL CONSIDERATIONS 287

16 Cut Points and Performance Characteristics for Anti-Drug Antibody Assays 289
Viswanath Devanarayan and Michael G. Tovey

17 Dilutional Linearity for Neutralizing Antibody Assays 309
David Lansky and Carrie Wager

PART VI ADAPTATION OF ANTI-DRUG ANTIBODY ASSAYS TO CHALLENGING CONDITIONS 319

18 Detection of Antibodies to Biopharmaceuticals in the Presence of High Levels of Circulating Drug 321
Arno Kromminga and Michael G. Tovey

19 Antibody Assays in Animal Research 331
Vera Brinks, Francesca Gilli, Melody Sauerborn, and Huub Schellekens

20 Immunogenicity of Microbial Digestive Enzymes for Oral Replacement Therapy in Pancreatic Exocrine Insufficiency 343
Claudia Berger and Uwe Niesner

PART VII NOVEL TECHNOLOGIES FOR THE QUANTIFICATION OF NEUTRALIZING ANTIBODIES 369

21 Measurement of Biologically Active Drug as an Approach to Detection of Anti-Drug Neutralizing Antibodies 371
Yao Zhuang and Shalini Gupta

22 A Novel One-Step Cell-Based Assay for Quantification of Neutralizing Antibodies to Biopharmaceuticals 383
Christophe Lallemand and Michael G. Tovey

INDEX 399

INTRODUCTION: A PERSPECTIVE

Michael G. Tovey

Recombinant biopharmaceuticals represent an important class of therapeutic agents, as reflected by sales of some $92 billion in 2009 [1]. The safety and efficacy of recombinant biopharmaceuticals can be severely impaired, however, by their immunogenicity. In addition to adversely affecting pharmacokinetics, pharmacodynamics, bioavailability, and efficacy, anti-drug antibodies (ADAs) can also cause immune complex disease, allergic reactions, and in some cases severe autoimmune reactions. It is widely accepted that injection of foreign proteins into humans can elicit an immune reaction leading to the production of antibodies that in some cases may neutralize the activity of the protein. Neutralizing antibodies (NAbs) block the biological activity of a biopharmaceutical either by binding directly to an epitope within or close to the active site of the protein or by binding to an epitope that prevents binding of the drug to a cell surface receptor. It is becoming increasingly apparent, however, that repeated injection of recombinant homologues of authentic human proteins, such as interferon beta (IFN-β) or erythropoietin (EPO), especially when aggregated or partially denatured, can result in a break in immune tolerance to self-antigens, leading to the production of ADAs. This is of particular concern in the treatment of chronic diseases, including certain forms of cancer and autoimmune or inflammatory diseases such as multiple sclerosis or rheumatoid arthritis. ADAs can result in the failure of the patient to respond to therapy and may even prove to be life threatening in the case of NAbs that cross-react with essential nonredundant endogenous proteins such as EPO or thrombopoietin [2, 3]. Drug-induced immunoglobulin IgE antibodies can also cause serious anaphylactic reactions [4]. ADAs can also persist for long periods after cessation of treatment, thereby limiting subsequent treatment with the

same drug [5]. Assessment of immunogenicity is therefore an important component of drug safety evaluation in both preclinical and clinical studies and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Immunogenicity is a complex phenomenon influenced by both drug-related factors, including molecular structure, glycosylation, and the presence of degradation products, aggregates, or impurities, and patient-related factors such as genetic makeup, age, gender, disease status, concomitant medication, and route of administration. Currently available techniques do not permit one to predict with a sufficient degree of accuracy, however, whether a product will be immunogenic and in which patients and at what point during treatment an immune response will occur.

The objective of this volume is to provide a single source of information both for those new to the field, seeking a clear understanding of the principal questions involved in the detection and quantification of antibodies to biopharmaceuticals, and for the experienced practitioner, seeking information on a specific topic. Each chapter outlines the principles of the topic covered and, when appropriate, provides sufficient background theory for a clear understanding of the subject together with practical information on how to approach each specific problem. This approach allows the information provided in this volume to be applied to well-established therapeutic proteins or classes of therapeutic proteins as well as to drugs in development or novel classes of molecules.

It is widely accepted by both regulatory agencies and industry alike that a risk-based strategy should be used to assess the potential immunogenicity of a biopharmaceutical. In Chapter 1, Eugen Koren, Erik Foehr, and Charles O'Neill describe a rational basis for the design of appropriate detection strategies and assays for antibodies to biopharmaceuticals. An approach is outlined for assessment of the antibody response to high-risk products such as a recombinant analogue of a nonredundant endogenous protein. Such an approach requires the development of a sensitive neutralization assay, frequent testing, and determination of cross-reactivity of anti-drug antibodies with the endogenous counterpart of the drug. An appropriate approach is also described for assessment of immunogenicity for low-risk products without an endogenous counterpart, where a less rigorous testing procedure may be appropriate. Consideration is also given to the development of risk-based strategies for assessment of the antibody response to multicomponent biopharmaceuticals and to biopharmaceuticals used in replacement therapy for genetic deficiencies.

The following three chapters describe the American, European, and Japanese regulatory perspectives on assessment of the immunogenicity of therapeutic proteins. It emerges from these chapters that, although differences in approach and emphasis certainly exist among the different regulatory authorities, there is nevertheless a large degree of consensus on the type of approach that should be adopted: namely, a risk-based approach that is clini-

cally driven, takes into account pharmacokinetic data, and uses appropriate screening and confirmatory assays for the detection of both binding and neutralizing ADAs

In Chapter 2, the first of the three chapters dealing with regulatory requirements, Susan Kirshner reviews US Food and Drug Administration (FDA) requirements for the assessment of the immunogenicity of a protein therapeutic, including the December 2009 FDA Draft Guidance for Industry [6]. The chapter outlines the current view of the risks of ADAs to safety and efficacy, factors that contribute to the development of ADAs, and strategies for controlling and managing an ADA response from a regulatory perspective.

In Chapter 3, Meenu Wadhwa and Robin Thorpe describe and analyze the implications of the European Medicines Agency (EMEA) Guideline on Immunogenicity Assessment [7], established by the Committee for Medicinal Products for Human Use (CHMP) of the EMEA, that came into effect in April 2008. Topics include factors that may influence the development of an immune response against a therapeutic protein, nonclinical assessment of immunogenicity and its consequences, development of assays for detecting and measuring immune responses in humans, immunogenicity and clinical development, and the establishment of a risk-based management plan. It is emphasized that the guidelines provide a general framework for a systematic and comprehensive evaluation of immunogenicity that can be modified as appropriate, case by case. It is also emphasized that evaluation of immunogenicity is an evolving process that continues for the whole life cycle of the drug and may involve postapproval clinical studies as part of pharmacovigilance surveillance.

In Chapter 4, Takao Hayakawa and Akiko Ishii describe the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) requirements for assessment of the immunogenicity of therapeutic proteins. This includes the PMDA requirements for the evaluation of the immunogenicity of biopharmaceuticals in preclinical and clinical studies and in postmarketing pharmacovigilance monitoring. Although a number of the biopharmaceuticals commercialized in Japan have previously been commercialized in Europe and North America, Takao Hayakawa and Akiko Ishii describe the example of anti-adalimumab antibodies, the incidence of which was substantially greater in the Japanese population than in Westerners, illustrating the fact that the incidence of immunogenicity of a therapeutic protein can vary from one population to another. The chapter also describes the approval process in Japan for recombinant human serum albumin (rHSA) produced in the yeast *Pichia pastoris*. The high dose of the product administered necessitated the reduction of process-derived impurities to as low a level as possible to ensure safety. Furthermore, the presence of anti-yeast IgE in some individuals due to exposure to yeast products in daily life, and hence the risk of severe hypersensitivity reactions in these individuals, led to mandatory testing for the presence of anti-pichia IgE prior to treatment. These examples illustrate how both ethnicity and dietary differences can influence the incidence of immunogenicity of therapeutic

proteins and the importance of taking such factors into consideration when testing biopharmaceuticals in different populations.

Monitoring patients for the presence of ADAs to biopharmaceuticals and correlating immunogenicity with clinical data are key factors in determining the safety of treatment and interpreting clinical data. Whether monitoring for ADAs is carried out in the context of either clinical trials or postmarketing surveillance, numerous samples will be generated that will require the establishment of appropriate screening and confirmatory assays. In Chapter 5, Klaus Bendtzen and Morten Svenson review the importance of using appropriate assays to detect ADAs in order to obtain clinically relevant data that can guide the clinician in the choice of treatment options. This is illustrated by reference to the development of screening assays for the detection of antibodies to the tumor necrosis factor alpha (TNF-α) antagonists currently in clinical use. The advantages of fluid-phase radioimmunoassays (RIAs) or fluid-phase enzyme immunoassays (EIAs) relative to the more common solid-phase enzyme-linked immunosorbent assays (ELISAs) are discussed. Thus, although ELISAs are sensitive and relative simply to use, they are subject to serum matrix effects and interference from the presence of residual drug in a sample. Bridging-ELISAs are less sensitive to the presence of residual drug but are subject to interference by rheumatoid factors or components of complements. Bridging ELISAs also fail to detect monovalent antibodies of the IgG4 subclass. On the other hand, while RIAs are less prone to false positive results due to nonspecific binding, or false negative results due to epitope masking, their application is restricted due to the use of radiolabeled probes.

In Chapter 6, Eric Wakshull and Daniel Coleman review the design of confirmatory assays, for anti-drug antibodies identified in an initial screening assay. The authors emphasize the importance of the use of a tiered strategy in which ADAs identified in a screening assay, designed to detect all ADAs, are further assayed in a confirmatory assay designed to determine their functional significance. Ideally, confirmatory assays should provide information different from that obtained in the initial screening assay. Contrary to conventional wisdom, the authors suggest that using the same assay format as that used in the screening assay, but with samples spiked with excess drug, can provide independent information while minimizing variation due to the use of different assay platforms. An additional advantage of such an approach is that it allows both screening and confirmatory assays to be run on the same plate. Confirmed positive samples can then be further characterized either in a titration assay to determine their potency or in a functional assay to determine their ability to neutralize the activity of the biopharmaceutical.

In Chapter 7, Florian Deisenhammer discusses the difficulty of detecting immunoglobulins and neutralization of biological activity in a single assay. He describes the use of pharmacodynamic parameters, particularly drug-induced biological markers, to quantify *in vivo* or *ex vivo* the activity of biopharmaceuticals such as interferons (IFNs) that are difficult to measure directly due to the very low levels present in the peripheral circulation using current treat-

ment regimens. Thus, IFN-induced gene products such as the myxovirus resistant protein A (MxA) that exhibit favorable pharmacodynamic characteristics are used widely as a biomarker of IFN activity. MxA expression is assessed either by ELISA, using a pair of monoclonal antibodies specific for the MxA protein, or by quantification of MxA mRNA using quantitative reverse transcription polymerase chain reaction (RT-PCR). The activity of IFN biomarkers such as MxA can also be determined directly in peripheral blood mononuclear cells as an indirect indication of the presence of anti-IFN NAbs. Although such an approach has the advantage of simplicity, a loss of IFN bioactivity does not necessarily correlate with the presence of NAbs.

Detection and quantification of NAbs has traditionally relied upon the use of cell-based assays. As essentially any readily measurable activity can be used as the basis for establishment of a cell-based assay for the quantification of NAbs against a particular biopharmaceutical, this has led to a wide diversity of assays. This diversity is illustrated in Chapter 8, where Anthony Meager outlines the principal types of cell-based assays used for the detection and quantification of NAbs to type I IFNs and to TNF-α antagonists. Cell-based assays for IFN-α or IFN-β range from various types of antiviral assays to quantification of the transcriptional activity of IFN-induced genes or detection of IFN-induced proteins, such as MxA, or the use of cell lines stably transfected with various reporter genes. Although IFN assays can be calibrated for quantifying IFN potency by using the appropriate World Health Organization (WHO) International IFN Standard, the polyclonal nature and variable composition of IFN NAbs—in terms of affinity, immunoglobulin class, and isotype composition as well as of epitope specificity, both between individuals and for a particular individual at different times during therapy—precludes the use of antibody standards for potency determinations. The use of cell-based assays to detect antibodies against TNF-α antagonists is confronted with the difficulties associated with the presence of high circulating levels of both free drug and drug–ADA soluble immune complexes in individuals treated with TNF-α antagonists. Provided such difficulties can be overcome, cell-based assays based on induction of apoptosis in TNF-α-sensitive cell lines can be used to quantify NAbs against TNF-α antagonists in an indirect NAb assay based on restoration of TNF-α-induced cytotoxicity. Although reference preparations for NAbs against TNF-α antagonists are not available, TNF-α activity can be calibrated using the WHO International Standard for TNF-α. Ligand binding assays using immobilized cells expressing noncleavable TNF-α may also provide a basis for the detection of NAbs against TNF-α antagonists [8].

In Chapter 9, Francesca Gilli and Antonio Bertolotto describe the use of real-time PCR for the quantification of anti-IFN-β NAbs based on measurement of IFN-β-induced MxA mRNA in interferon-sensitive cells. The advantages of this method relative to the measurement of MxA protein levels or the measurement of interferon antiviral activity using the cytopathic effect (CPE) assay are discussed in terms of savings in assay time, labor,

and materials and quality assurance. The authors also provide an analysis of the relative advantages of the use of SYBER Green to label double-stranded DNA or the use of fluorescent probes to quantify PCR products such as those used in the TaqMan® system. The choice of suitable housekeeping genes or ribosomal RNA for the normalization of results is also discussed, as are different strategies for the quantification of results, such as standard curves or the comparative cycle threshold (Ct) method. The relative advantages of different methodologies, such as manual and semiautomated methods for RNA extraction or direct analysis without RNA purification, or the use of random hexamers or oligo d(T)16 for retrotranscription of RNA, are also outlined.

Detection and quantification of NAbs has traditionally relied upon the use of cell-based assays that are difficult to standardize and subject to interference from the presence of residual drug in a sample. In Chapter 10, Bonnie Wu, George Gunn III, and Gopi Shankar discuss the use of competitive ligand-binding assays for the quantification of biopharmaceuticals, such as certain antagonistic monoclonal antibodies, that exert their action by binding to a soluble ligand, thereby preventing the ligand from interacting with a receptor or other molecule on the surface of target cells. In such instances competitive ligand-binding assays, based on the use of an electrochemiluminescence (ECL) detection system, offer a number of advantages for the quantification of NAb levels, including a wide dynamic range and a relatively high degree of tolerance to the presence of residual drug in samples. The use of an ECL ELISA-based format commercialized by Meso Scale Design is described. The authors also discuss the relative advantages of using a direct format, in which the presence of ADAs in a sample prevents ruthenium-labeled ligand from binding to immobilized drug, or indirect formats based on drug-mediated inhibition of ligand–receptor binding. Examples are also given of the use of cell-based binding assays in which the presence of ADAs in a sample inhibits the binding of labeled drug to the receptor expressed on the surface of immobilized cells. Although such assays are undoubtedly useful, as indicated by their widespread adoption, they do not, however, completely replace the need for cell-based assays. Inhibition of ligand binding does not always equate to the neutralization of biological activity. For example, monoclonal antibodies to the type I IFN receptor have been described that neutralize biological activity without inhibiting binding of IFN to its receptor [9].

Surface plasmon resonance (SPR) can be used to detect ADAs present in a serum sample by measurement of binding to the biopharmaceutical immobilized on a sensor chip. Real-time SPR-based instruments such as the Biacore® biosensor are particularly useful for the detection of low-affinity antibodies produced during the initial stages of an immune response. The immunoglobulin isotype composition of the ADAs' response to a biopharmaceutical can be readily determined using SPR. The Biacore instrument also allows antibodies to be characterized for binding to different domains of the biopharmaceutical or for cross-reactivity with an endogenous protein. In Chapter 11, Steven Swanson and Daniel Mytych describe in detail the use of the Biacore biosen-

sor for characterization of ADAs during the different stages of the development of a biopharmaceutical.

In Chapter 12, Jörgen Dahlström and Lennart Venemalm review current assays for the detection of IgE antibodies to biopharmaceuticals. IgE antibodies specific for epitopes present upon biopharmaceuticals can cause serious adverse reactions, including anaphylaxis, even upon the first treatment exposure. Pre-existing antibodies against similar antigens may cross-react with a biopharmaceutical and thus a medical history of anaphylactic reactions may indicate that a patient is at a higher risk of developing serious allergic reactions against the drug. IgE antibodies are present at low concentration in human serum and sensitive assays are required for the detection IgE ADAs.

In Chapter 13, Daniel Kramer reviews the technical and regulatory considerations involved in the standardization and validation of monoclonal-antibody-based immunoassays such as ELISAs, RIAs, and ECL for the detection of anti-drug antibodies. The author emphasizes the importance of establishing a validated assay that can detect clinically relevant events during the whole course of development of a biopharmaceutical, including postmarketing studies. A strategy and key performance criteria are outlined for the standardization and validation of immunoassays, based upon a tiered testing approach in which samples are first screened for the presence of ADAs using a validated immunoassay; samples that test positive are then further tested in a validated confirmatory assay. Additional analyses, including potency determinations, and/or assays for the presence of neutralizing antibodies, are determined on a case-by-case basis based upon perceived risk to patient safety. Such a strategy ensures an efficient assessment of immunogenicity with optimal safety evaluation, and an efficient use of resources.

In Chapter 14, Deborah Finco-Kent and Amy Grenham review the technical challenges and regulatory considerations involved in the standardization, optimization, and validation of cell-based assays for the detection of neutralizing anti-drug antibodies. The authors outline a tiered testing approach for the assessment of immunogenicity and emphasize the importance of the determination of the neutralizing capacity of ADAs where NAbs potentially pose a risk to patient safety or drug efficacy. The authors point out that it is critical to have a validated assay in place that can detect clinically relevant events that may be related to neutralizing ADAs and outline strategies and key performance parameters for the development, optimization, and validation of cell-based assays to measure NAbs. The authors also discuss the challenges posed by the development of standardized assays for assessment of the immunogenicity of biopharmaceuticals across a product class. Such standardized assays would allow the incidence of NAbs to be compared for different products within a class and allow a better understanding of the correlation between NAb development and clinical effects.

In Chapter 15, Sidney Grossberg, Yoshimi Kawade, and Leslie Grossberg describe a standardized approach to bioassay design for the quantification of neutralizing antibodies to biopharmaceuticals. The authors describe the

theoretical and experimental basis for the use of 10-fold reduction units (TRU) as a measure of NAbs to cytokines such as type I interferons or interleukin-6. This methodology is generally applicable to a wide range of protein effector molecules, and the authors illustrate the use of this methodology applied both to the commonly employed constant antigen method for quantifying NAbs and to the constant antibody method. In the first method a given concentration of an antigen is mixed with serial dilutions of serum to determine the lowest dilution that neutralizes a constant proportion of antigen. In contrast, the constant antibody method is based upon mixing a given dilution of serum with increasing concentrations of antigen. Theoretical neutralization curves based on the established model of the type I interferon antibody reaction are depicted in terms of experimentally observable quantities. As predicted by theoretical studies, the constant antibody method extends the lower limits of antibody detection.

An essential part of assay development is the determination of the assay's performance characteristics, including sensitivity, precision, and dilution linearity. In Chapter 16, Viswanath Devanarayan and Michael Tovey review the establishment of cut points and performance characteristics for anti-drug antibody assays. The authors emphasize the importance of establishing appropriate performance characteristics for assays in order to obtain reliable immunogenicity data. The determination of the different types of assay precision such as sensitivity, low positive control, precision, and in-study acceptance criteria are outlined as well as the establishment of the most appropriate type of cut point for both screening and confirmatory assays.

In Chapter 17, David Lansky and Carrie Wager discuss the importance of assessing dilution linearity for NAb assays in order to establish acceptable limits of bias. The study of dilution linearity allows the effect of sample dilution on the precision and bias of the response measured in the assay to be determined experimentally. Most cell-based assays are characterized by a nonlinear sigmoid dose–response relationship best defined by a four-parameter logistic nonlinear regression model. Although NAb levels are often expressed as a titer, the use of sample-specific ED_{50}s, which provide an estimate of the NAb concentration required to inhibit 50% of the activity of the biopharmaceutical, is preferable since comparisons among them have low variance and they are invariant with respect to slope for curves having similar asymptotes. The authors define five sources of error in the measurement of a NAb assay response (measurement bias, truncation bias, population dilution bias, specimen-specific dilution bias, and other sources of bias) and outline procedures for evaluating the similarity of the dose–response curves for different samples, establishing dilution linearity for NAb assays

In Chapter 18, Arno Kromminga and Michael Tovey discuss the challenges posed by the detection of anti-drug antibodies in the presence of residual drug. This is of particular concern in the case of therapeutic monoclonal antibodies, which are often administered at high doses and are cleared slowly from the peripheral circulation. Accurate quantification of ADA levels is also rendered

difficult by the inability to detect ADA bound to drug in the form of soluble immune complexes. The relative drug tolerance of different types of ADA assays is reviewed, together with procedures for determination of the drug tolerance of a particular assay. Each assay methodology is discussed only briefly with respect to its relative drug tolerance since each assay format is described in detail in the other chapters in this volume.

In Chapter 19, Vera Brinks, Francesca Gilli, Melody Sauerborn, and Huub Schellekens describe the development of assays for the quantification of neutralizing antibodies to human biopharmaceuticals in immune tolerant transgenic mice and the use of these assays to study factors such as changes in drug formulation that can affect the immunogenicity of drugs based on recombinant analogues of human proteins. The authors describe the development of a real-time RT-PCR bioassay, based on the expression of the interferon-induced protein MxA, for the quantification of anti-IFN-β NAbs in mice immune tolerant for human interferon beta. The results of this assay correlate with BAB titers obtained by ELISA.

In Chapter 20, Claudia Berger and Uwe Niesner outline an approach for the detection and quantification of antibodies to mixtures of recombinant enzymes administered as oral replacement therapy in the treatment of pancreatic exocrine insufficiency, a serious and life-threatening condition caused by underlying diseases such as cystic fibrosis and chronic pancreatitis. The authors outline an immunogenicity risk assessment strategy for orally administered microbial digestive enzymes that follows the same overall strategy as that used for parenteral biopharmaceuticals. The potential impact of an adverse immune response is determined for each individual product based on an assessment of a number of product-related and patient-related factors. A preclinical and clinical immunogenicity testing program including assay design and sample timing is then established for each individual product. Although microbial digestive enzymes can for the most part be classified as low-risk products due to their local action in the gastrointestinal tract, trace systemic bioavailability, and low sequence homology to endogenous counterparts, nevertheless some patients may develop an ADA response over time and it is important to have an adequate immunogenicity testing program in place for every enzyme present in a product. Characterization of IgE-mediated responses should also be an integral part of the immunogenicity assessment program for such products.

In Chapter 21, Yao Zhuang and Shalini Gupta describe a novel drug-quantification assay for the detection of anti-drug neutralizing antibodies. The assay is based upon a two-stage approach. The first step involves quantification of the bioactivity of circulating drug present in samples that have tested positive for ADAs in an immunoassay. It is expected that the bioactivity of circulating drug will be reduced in samples containing clinically relevant levels of neutralizing antibodies. In the second step the loss of recovery of the bioactivity of a given quantity of added drug is determined. The drug-quantification assay provides a number of advantages relative to conventional NAb

assays, including enhanced sensitivity, tolerance to the presence of residual drug, and applicability to a large range of biopharmaceuticals.

In Chapter 22, Christophe Lallemand and Michael Tovey describe a novel one-step method for the quantification of anti-drug neutralizing antibodies based on the use of engineered reporter cells. The reporter cells have been co-transfected with the firefly luciferase reporter gene under the control of a drug-responsive promoter together with a second construct consisting of an expression vector for the drug of interest together with a second reporter gene, *Renilla* luciferase, transcribed from a common inducible promoter. Expression of the two reporter genes is determined sequentially in the same sample. Drug concentration is quantified rapidly and with a high degree of precision by quantifying *Renilla* luciferase expression by the measurement of measurement of light emission. Secreted drug will attach to specific cell-surface receptors on the reporter cells, activating the drug-regulated reporter gene. Anti-drug antibody concentration is determined by the constant antibody method from the ratio of light emission from the two reporter genes before and after addition of a serum sample containing ADAs. The advantages of this system relative to conventional assays for the quantification of NAbs against human type I IFNs and the TNF-α antagonists infliximab, adalimumab, and etanercept are discussed. Advantages include the elimination of assay variation due to variations in cell number, the absence of sample or reagent manipulation, and the reduction of serum matrix effects to a minimum.

Assessment of immunogenicity is an important component of the evaluation of drug safety in both preclinical and clinical studies and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Assessment of immunogenicity, and in particular detection and quantification of anti-drug antibodies, is evolving rapidly in regard to both technological developments and the expectations of regulatory authorities. This is reflected by the growing number of white papers on the subject and the recent publication of guidance from the US and EU regulatory authorities. Initiatives are also under way for the establishment of international standards for certain human anti-drug antibodies [10]. The objective of the proposed volume is to provide insight into the design, optimization, and qualification of assays, the establishment of sampling strategies, the choice of appropriate assay endpoints, and data analysis for the detection and quantification of antibodies to biopharmaceuticals. It is hoped that the availability of a single volume covering all the principal aspects of the detection and quantification of antibodies, including a review of the expectations of regulatory authorities, will facilitate a more uniform approach to the assessment of immunogenicity of biopharmaceuticals.

REFERENCES

1. R & D Pipeline News, La Merie Business Intelligence, March 10, 2010 (www.lamerie.com).

2. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.
3. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
4. Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., et al. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3-galactose. *N Engl J Med*, *358*, 1109–1117.
5. Phillips, J.T. (2010). Interferon neutralizing antibodies in multiple sclerosis: a new perspective. *Arch Neurol*, *67* (4), 386–387. [Comment on "Clinical effect of neutralizing antibodies to interferon beta that persist long after cessation of therapy for multiple sclerosis," by van der Voort, L.F., Gilli, F., Bertolotto, A., Knol, D.L., Uitdehaag, M.J., Polman, C.H., Killestein, J. (2010). *Arch. Neurol*, *67* (4), 402–409.]
6. Food and Drug Administration (FDA). (2009). Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins. Center for Drug Evaluation and Research, Rockville, MD. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.PDF
7. European Medicines Agency (EMEA). (2007). Immunogenicity assessment of biotechnology-derived therapeutic proteins. Committee for Medicinal Products for Human Use (CHMP). Available at http://www.emea.europa.eu/pdfs/human/biosimilar/1432706en.pdf
8. Loyet, K.M., Deng, R., Laing W.C., Wu, Y., Lowman, H.B., DeForge, L.E. (2009). Technology comparisons for anti-therapeutic antibody and neutralizing antibody assays in the context of an anti-TNF pharmacokinetic study. *J Immunol Methods*, *345*, 17–28.
9. Novick, D., Nabioullin, R.R., Ragsdale, W., McKenna, S., Weiser, W., Garone, L., Burkins, C., Kim, S.H., Rubinstein, M., Tepper, M.A., Arulanandam, A.R. (2000). The neutralization of type I IFN biologic actions by anti-IFNAR-2 monoclonal antibodies is not entirely due to inhibition of Jak-Stat tyrosine phosphorylation. *J Interferon Cytokine Res*, *20*, 971–982.
10. World Health Organization (WHO). (2010). Available at www.who.int/biologicals/…/WHO_BS10.2153_2010-New_ref_materials.pdf

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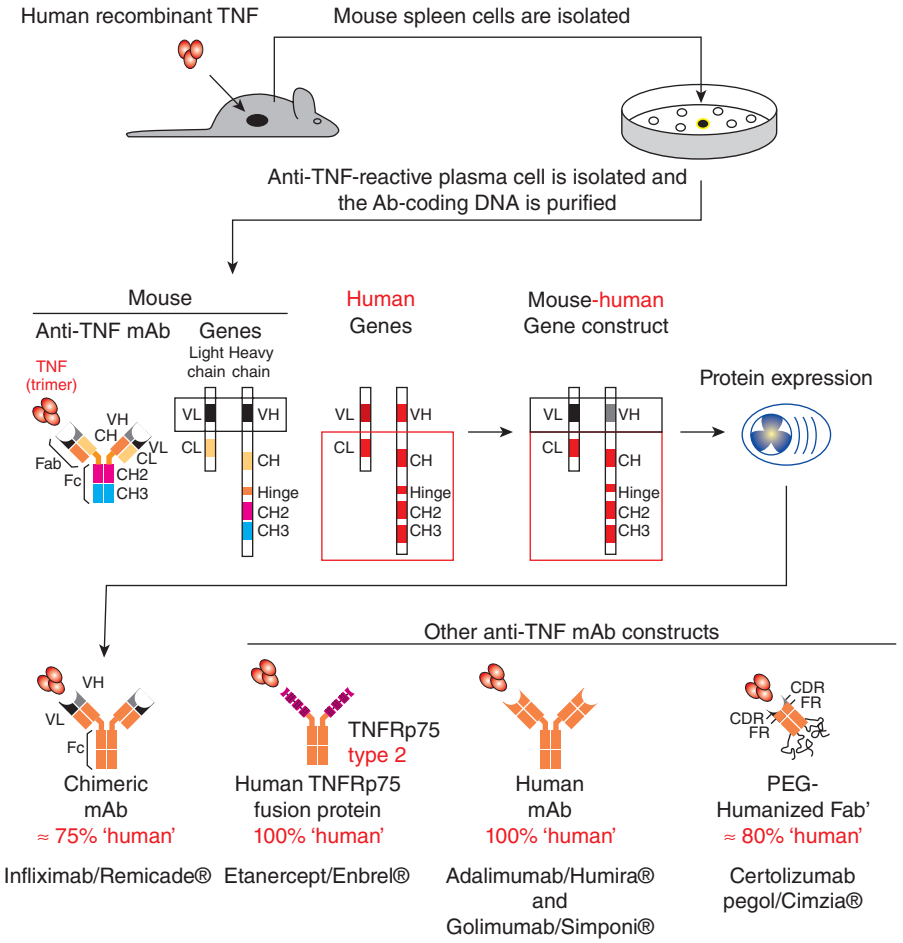


Fig. 5.1 Genetically engineered anti-TNF immunoglobulin constructs.

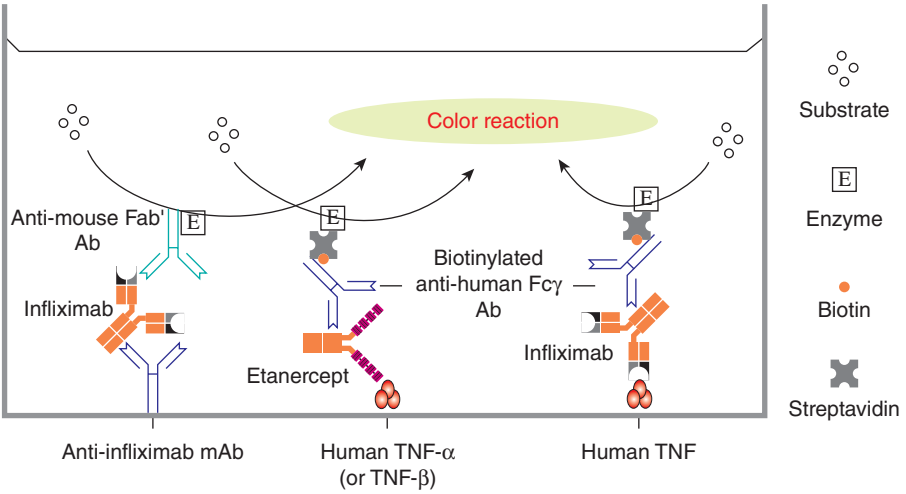


Fig. 5.2 ELISAs for TNF-inhibitors.

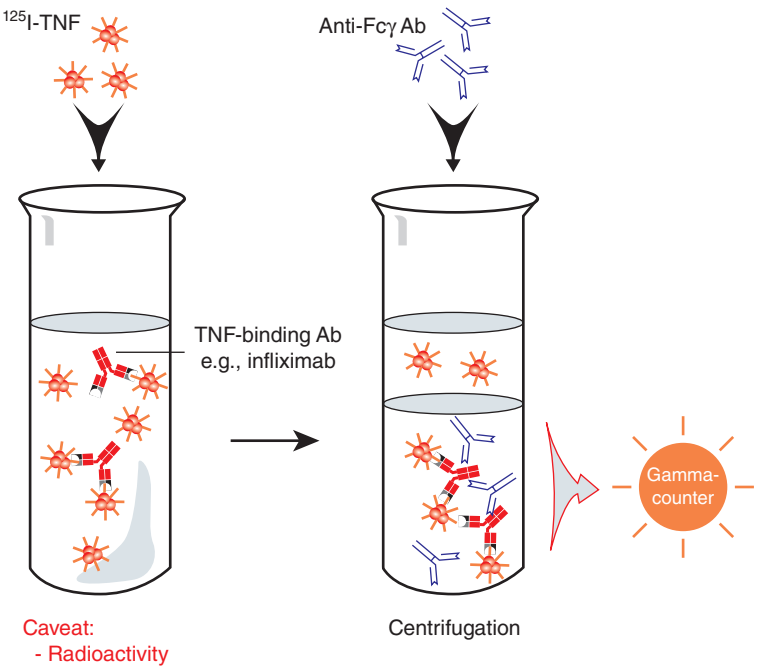
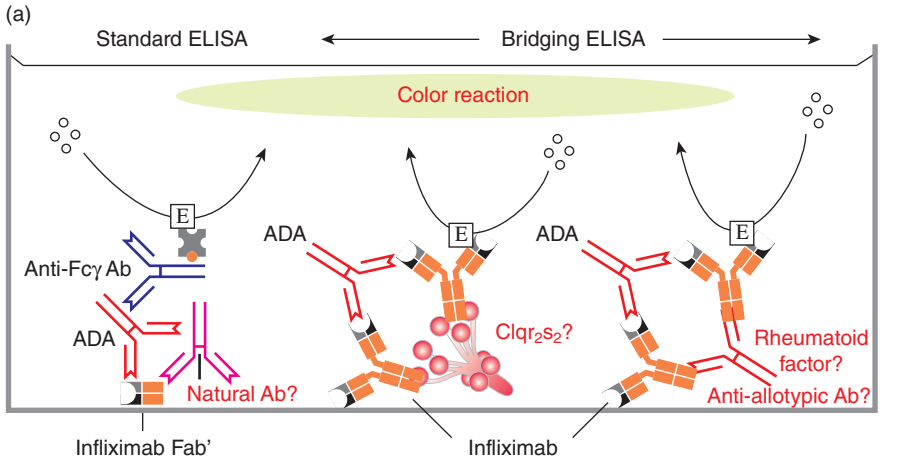
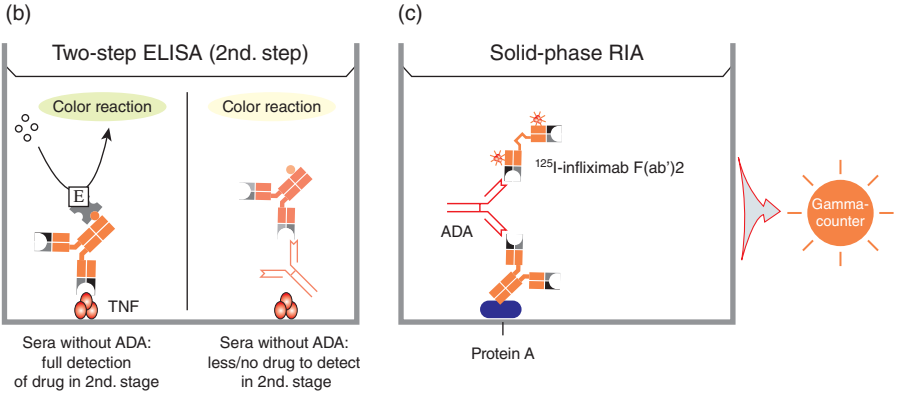


Fig. 5.3 Fluid-phase RIA for TNF-inhibitors.



Caveats:
- Does not detect ADA to drug Fc
- May detect natural Ab to denatured FAb'

- Does not detect IgG4 ADA
- Cannot detect low-level ADA
- May detect rheumatoid factor and anti-allotypic Ab
- May detect complement components



Caveats:
- Cannot quantify ADA in the presence of drug

Caveats:
- Does not detect IgG4 ADA
- Does not detect ADA to drug Fc
- Not applicable to other than IgG1- and IgG2-derived drugs

Fig. 5.5 Various techniques for the measurement of ADA to TNF-inhibitors.

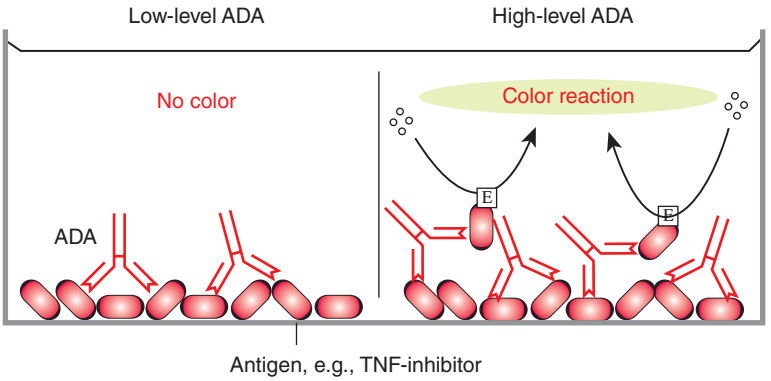


Fig. 5.6 Inability of bridging-ELISAs to assess low-level ADA.

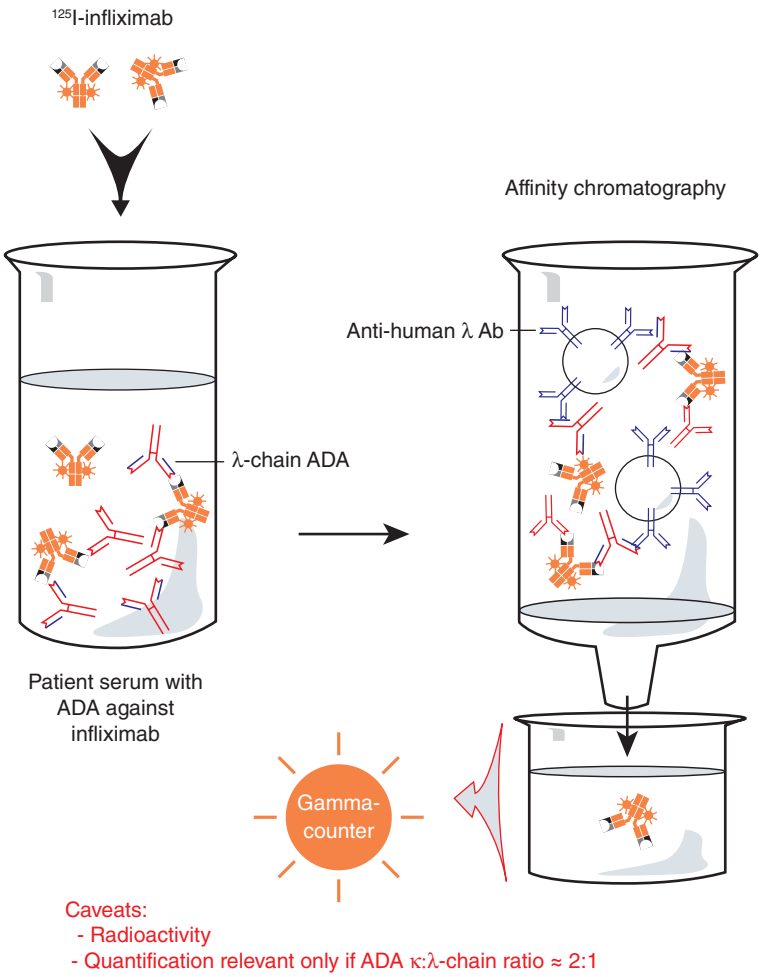


Fig. 5.7 Fluid-phase RIA for ADA to TNF-inhibitors.

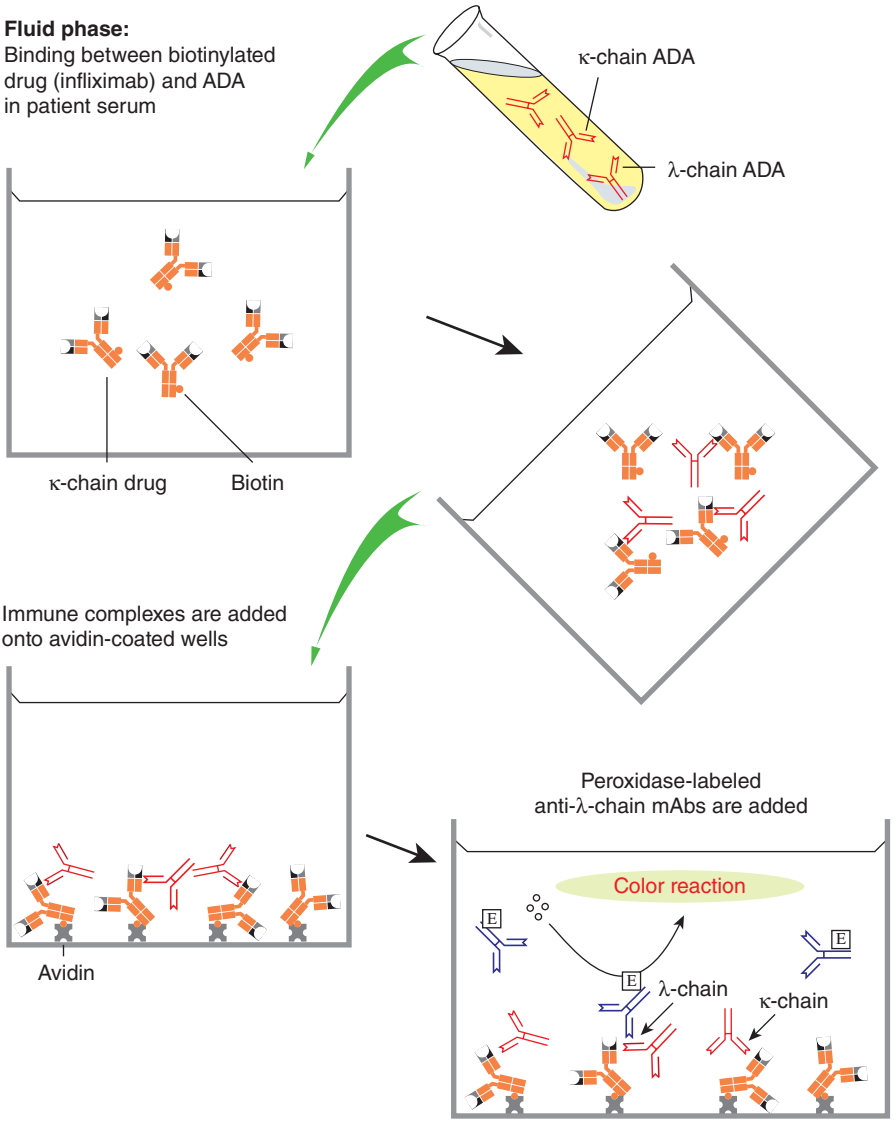


Fig. 5.9 Fluid-phase EIA for ADA toTNF-inhibitors.

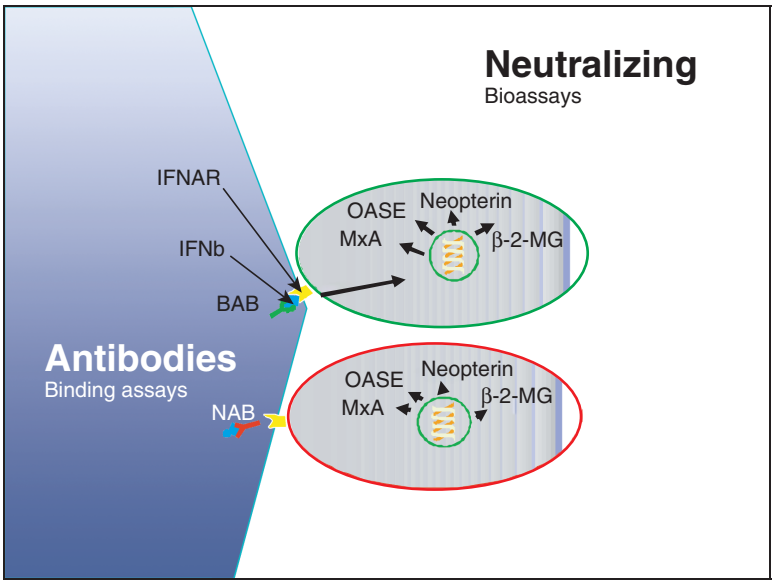


Fig. 7.1 Illustration of the dichotomy of neutralizing-antibody testing. (see text for full caption).

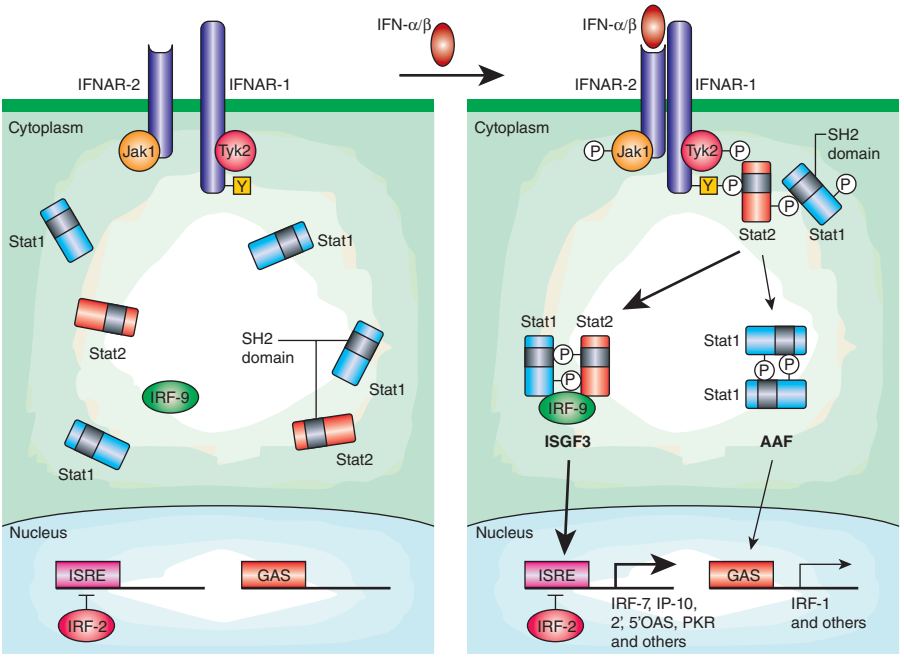


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Fig. 7.2 Type I interferon transmits signals through its homologous receptor complex IFNAR, which is composed of at least two units, IFNAR-1 and IFNAR-2. (see text for full caption).

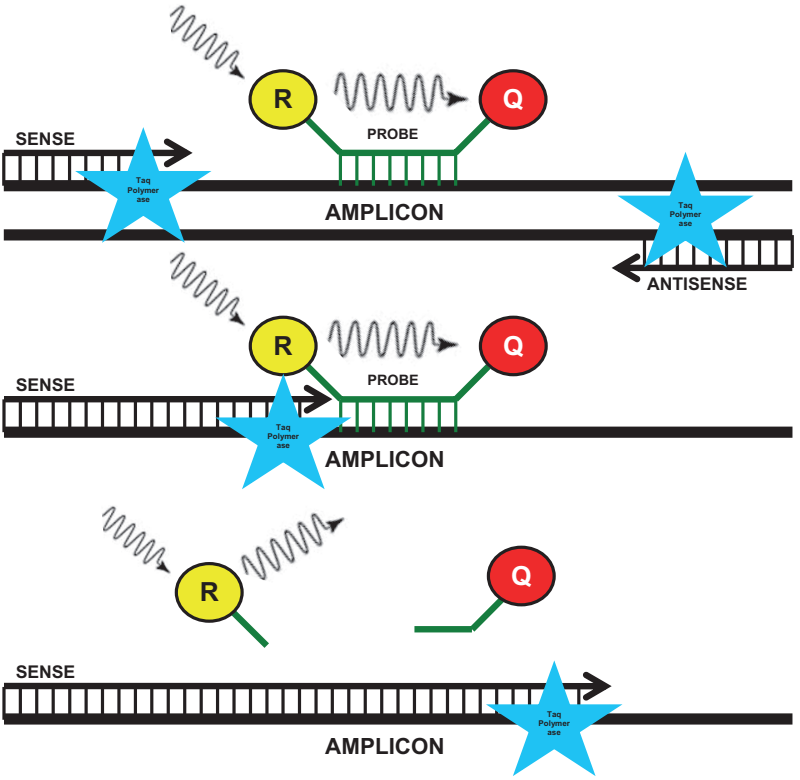


Fig. 9.1 TaqMan probe chemistry mechanism. TaqMan probes consist of a reporter (R), that is, a fluorophore covalently attached to the 5′-end of the oligonucleotide probe, and a quencher (Q) at the 3′-end. Several different fluorophores [e.g., 6-carboxy-fluorescein, (FAM), or tetrachlorofluorescin (TET)] and quenchers [e.g., tetramethyl-rhodamine (TAMRA) or dihydrocyclopyrroloindole tripeptide *minor groove binder* (MGB)] are available. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source via fluorescence resonance energy transfer (FRET). (A) As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. (B) TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5′ to 3′ exonuclease activity of the polymerase degrades the probe that has annealed to the template. (C) Degradation of the probe releases the fluorophore (R) from it and breaks the close proximity to the quencher (Q), thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time RT-PCR thermal cycler is directly proportional to the fluorophore released and the amount of cDNA template present in the PCR.

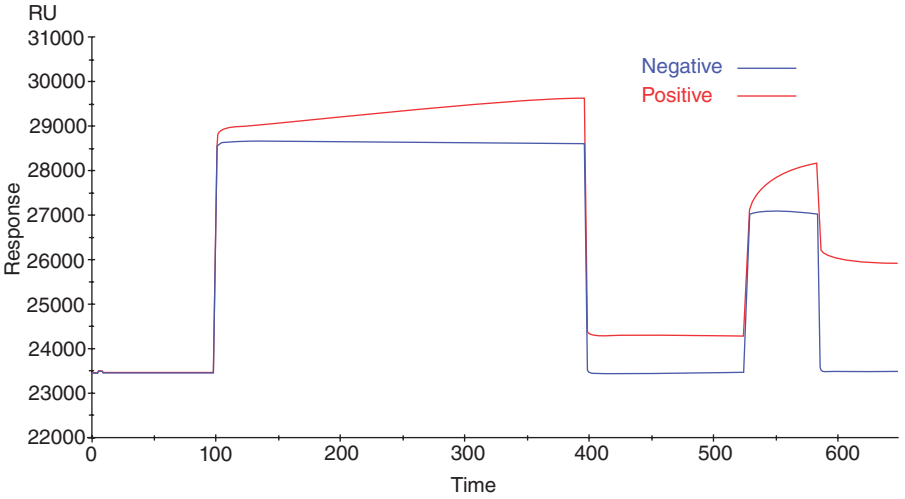


Fig. 11.3 A typical sensorgram from a positive and a negative sample. The positive sample demonstrates sample binding as well as binding of the confirmatory reagent.

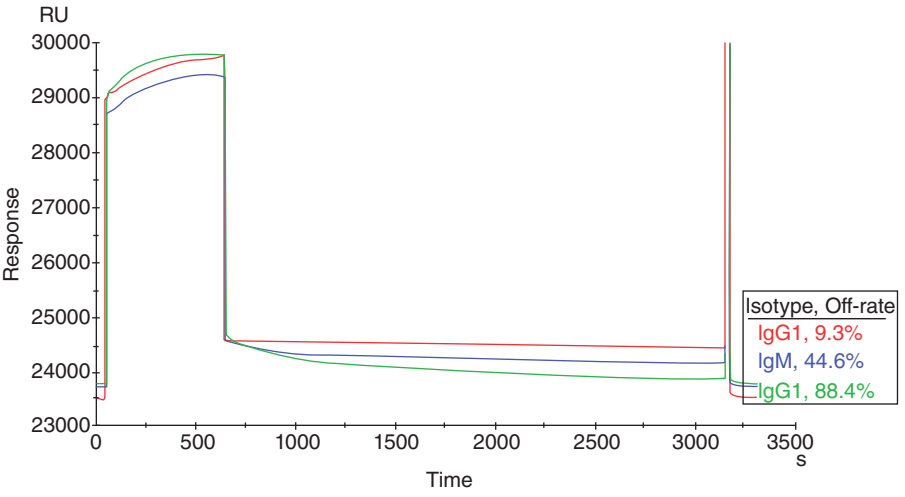


Fig. 11.4 This sensorgram illustrates differences in dissociation observed from antibodies with high, moderate, and low affinity. The low-affinity (rapidly dissociating) antibody is shown in green and the high affinity antibody is shown in red.

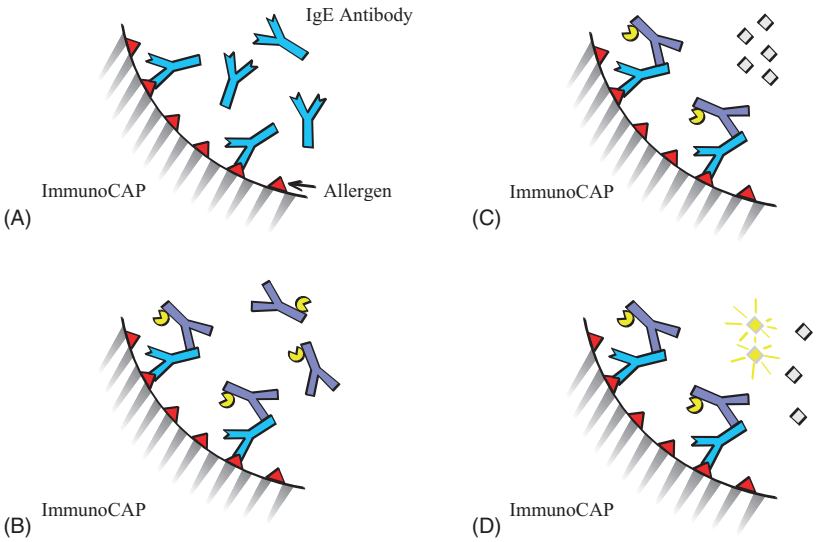
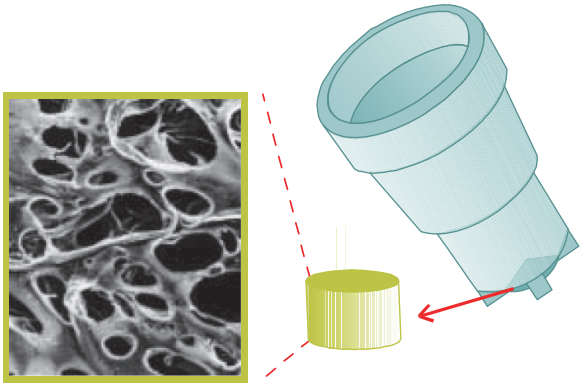


Fig. 12.1 Principle of the assay procedure. (A) The allergen of interest, covalently coupled to ImmunoCAP, reacts with the specific IgE in the patient sample. (B) After washing away nonspecific IgE, enzyme-labeled antibodies against IgE are added to form a complex. (C) After incubation, unbound enzyme–anti-IgE complex is washed away and the bound complex is then incubated with a developing agent. (D) After stopping the reaction, the fluorescence of the eluate is measured. The higher the fluorescence, the more specific IgE is present in the sample.



L. Sevéus & A. Sandell, 1992

Fig. 12.2 A solid phase with high binding capacity enabling the presence of an excess amount of antigen.

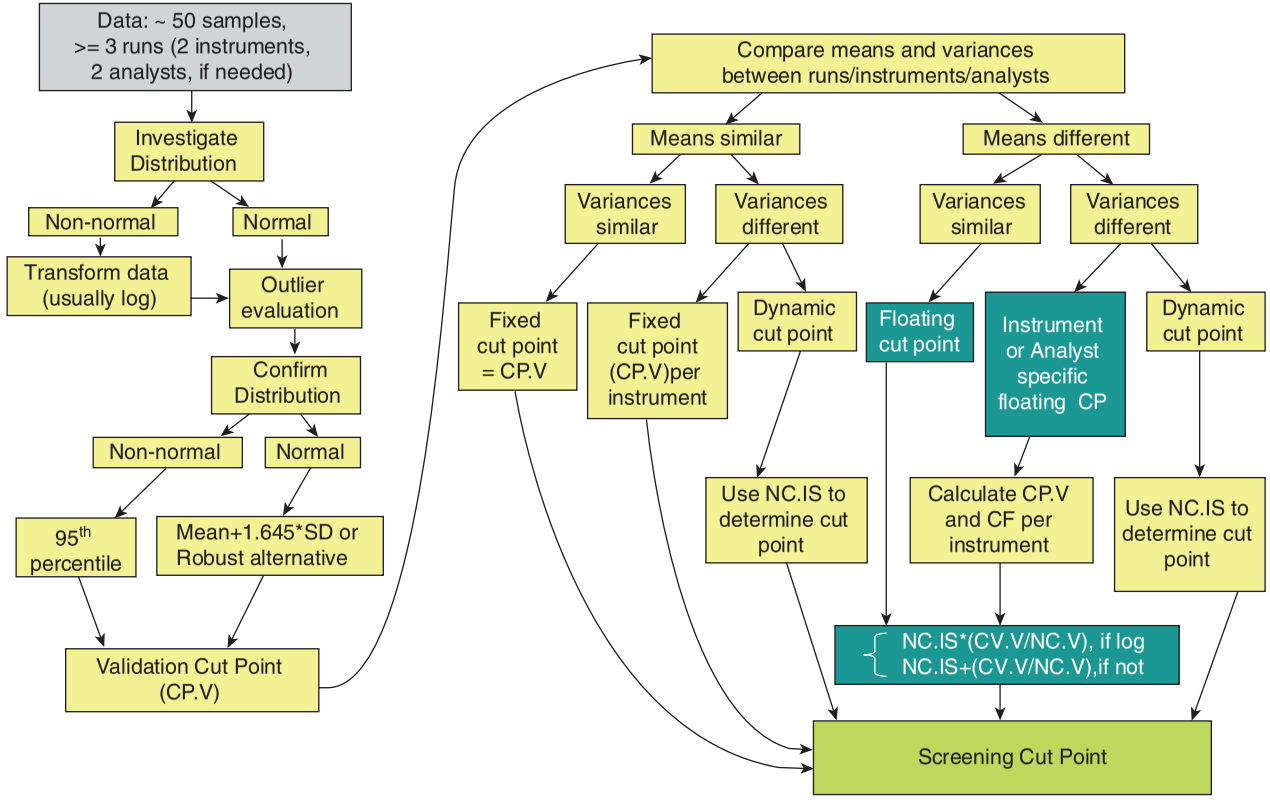


Fig 16.1 Flow scheme for screening-cut-point evaluation (NC.V = negative control from validation runs; NC.IS =negative control from in-study run).

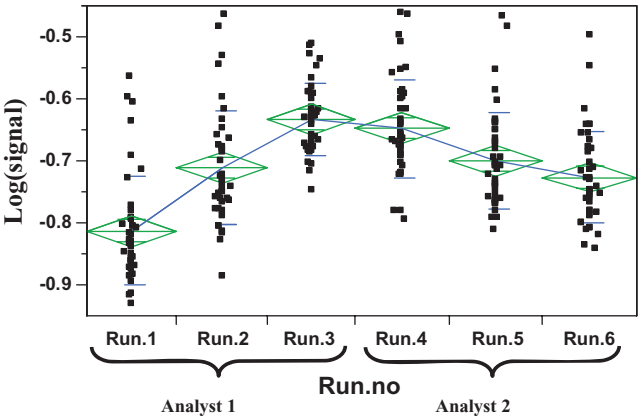


Fig. 16.2 Comparison of means and variances between assay runs; assay run means are significantly different ($p < 0.0001$) and variability is not significantly different (Levene's test, $p = 0.37$), thus a floating-cut-point method should be used.

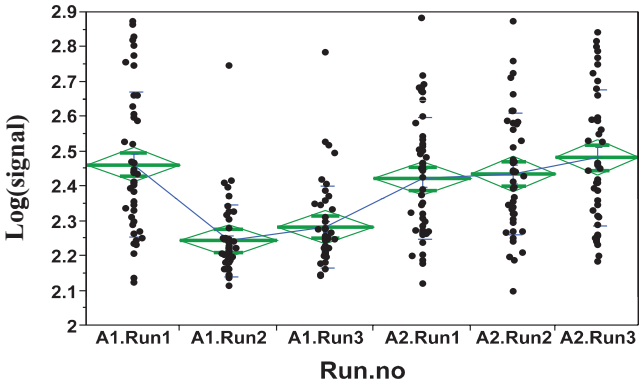


Fig. 16.3 In the axis labels, A1 and A1 refer to the two analysts, and Run1, Run2, and Run3 refer to the three runs performed by each of these two analysts. The means are significantly different ($p < 0.0001$) and variability is significantly different (Levene's test, $p < 0.001$), thus the data suggest using a dynamic cut-point; however, a floating cut point can be used in some cases (see text for details).

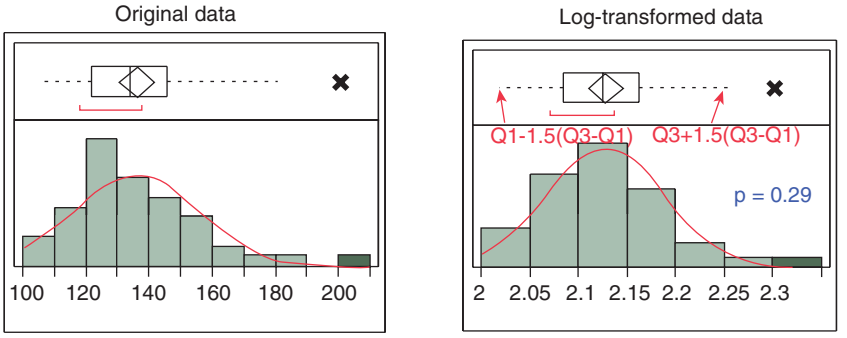


Fig. 16.4 Evaluation of distribution and outliers for data from screening-cut-point experiment (distribution of data from 48 subjects, averaged from 6 runs). Log-transformed data are relatively less non-normal. Subsequent analysis is therefore conducted in log-scale. This also illustrates the criteria for identifying outliers.

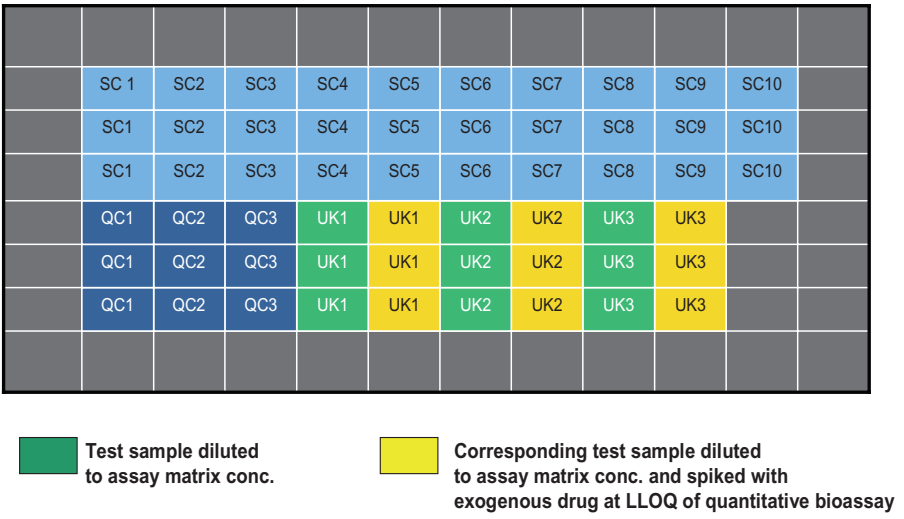

SC 1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
SC1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
SC1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
Test sample diluted to assay matrix conc.
Corresponding test sample diluted to assay matrix conc. and spiked with exogenous drug at LLOQ of quantitative bioassay

Fig. 21.4 A representative plate map that may be employed for conducting a DQ-NAb assay. SC1 to SC10 represent a multipoint drug standard curve prepared in undiluted target species serum. QC1–QC3 represent independently prepared quality control samples used to accept the performance of the drug standard curve. UK1–UK3 represent test samples for NAb analysis. The number of standard curve points and the replicates per point depend on the dynamic range and performance of the standard curve used in the assay.

PART I

RISK-BASED STRATEGIES

1

PRINCIPLES OF RISK ASSESSMENT AND MONITORING OF ANTIBODY RESPONSES TO BIOPHARMACEUTICALS

Eugen Koren, Erik Foehr, and Charles A. O'Neill

1.1. RISK ASSESSMENT

The first step in establishing a risk-based strategy for detection and characterization of antibodies to biopharmaceuticals is risk assessment. Ideally, risk assessment comprises an objective evaluation of risk factors that are clearly defined and understood. Assessing the risk of antibody response to biopharmaceuticals can be difficult because multiple factors contribute to the immunogenicity of therapeutic proteins: for example, structural properties; impurities and contaminants; formulation; the route and frequency of administration; dose level and duration of treatment; and patient health and genetic background.

Nevertheless, industry-wide experience with biopharmaceuticals over the last 25 years provides the basis for the design and validation of anti-drug antibody (ADA) methods [1–3] as well as rational, risk-based strategies for ADA monitoring during development and postapproval administration of biopharmaceuticals [4]. Clinical sequelae caused by antibodies directed against biopharmaceuticals can range from no apparent or mild side effects to

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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diminished efficacy, immune complex mediated symptoms, allergic reactions, or even autoimmune syndromes.

The first, and probably most important, step in risk assessment of a biopharmaceutical is to consider its type and its mechanism of action. If the biopharmaceutical is a molecule that has a nonredundant endogenous counterpart responsible for a vital physiological function, a great deal of caution should be taken. It has been shown that autoimmune-like syndromes can develop in patients with ADAs capable of neutralizing the biological activity of both the drug and its endogenous counterpart [5, 6]. These autoimmune-like syndromes induced by biopharmaceuticals can be life threatening and could persist for prolonged periods of time [7], as a continuous cycle of antibody production is maintained by the endogenous molecule even after treatment with the biopharmaceutical is stopped. In addition to the molecular nature of a biopharmaceutical and its mechanism of action, there are other important factors that should be considered during the risk assessment, such as the route and frequency of drug administration as well as the patient's immune status. It is generally accepted, and has been demonstrated in some cases, that in head-to-head clinical comparison the subcutaneous route is more immunogenic than the intramuscular or intravenous route [8].

1.2. HIGH-RISK BIOPHARMACEUTICALS

One of the most illustrative examples of a high-risk biopharmaceutical is recombinant human thrombopoietin (rhTPO). According to the criteria outlined in Section 1.1, thrombopoietin belongs to the category of high-risk molecules because it represents a nonredundant, endogenous growth factor that regulates a vital physiological function. In one development program, an antibody monitoring strategy as well as dosing regimen was designed on the basis of a rigorous risk assessment. During clinical development, administration of rhTPO was limited to immunocompromised cancer patients. In several clinical studies, rhTPO was dosed exclusively via the intravenous route. This dosing scheme was also accompanied by extensive antibody monitoring, utilizing three different assays: (a) a screening enzyme-linked immunosorbent assay (ELISA) for antibodies that bound to the full-length rhTPO molecule; (b) an ELISA for antibodies binding to the amino-terminal (bioactive) portion of the molecule; and (c) an ELISA for antibodies that inhibit binding of rhTPO to its receptor. These assays were used sequentially in a contingent fashion. If positive in assay (a), the sample was analyzed in assay (b); then if positive in assay (b), the sample was tested in assay (c). In addition, samples positive in all three ELISAs were analyzed in a human megakaryocyte proliferation assay for antibodies capable of neutralizing rhTPO's biological activity. These assays were carried out in real time (weekly) to provide clinicians with timely information on the occurrence and type of antibodies so that rhTPO administration could be stopped if the presence of neutralizing antibodies was detected. No

neutralizing antibody–related adverse effects were observed in any of the multiple clinical studies utilizing intravenous rhTPO administration [9].

In another development program, a truncated form of recombinant thrombopoietin (TPO), megakaryocyte growth and development factor (MGDF), was subcutaneously administered to immunocompromised cancer patients and to healthy human subjects. Neutralizing anti-MGDF antibodies were detected in 0.6% and 4% of these subjects, respectively. Long-lasting severe thrombocytopenia was associated with anti-Tpo neutralizing antibodies in some subjects [7, 10]. Differences in clinical outcomes observed in these two thrombopoietin development programs underscore the importance of a careful risk assessment combined with a comprehensive ADA monitoring strategy and an appropriate dosing strategy.

Another example of a high-risk biopharmaceutical is recombinant human erythropoietin (rhEPO). It has been shown that neutralizing antibodies directed against rhEPO can induce pure red cell aplasia (PRCA) [6, 11]. In a number of kidney dialysis patients who were treated with rhEPO and who developed PRCA, ADA response was characterized by the surface plasmon resonance (SPR) method combined with a cell-based bioassay. The SPR assay allowed for determination of the overall ADA level, isotypes, and affinity. High levels of the high-affinity immunoglobulins IgG1 and IgG4 were found. The same samples also inhibited *in vitro* cell proliferation induced by the rhEpo, demonstrating the presence of neutralizing antibodies [12]. In contrast, low-affinity, non-neutralizing IgM ADAs have been detected occasionally in patients with no signs of PRCA (Amgen, data on file). These data indicate that the high levels of high-affinity ADAs with neutralizing activity are more likely to induce PRCA.

1.3. LOW- TO MODERATE-RISK BIOPHARMACEUTICALS

Therapeutic humanized and human antibodies represent lower risk because ADAs directed against these biopharmaceuticals are not likely to cross-react with endogenous molecules and cause autoimmune syndromes. The risks of ADAs to therapeutic antibodies have been associated primarily with loss of efficacy. For example, in patients with Crohn's disease treated with infliximab, the chimeric monoclonal antibody to tumor necrosis factor alpha (TNF-α), the median therapeutic response to this drug was significantly shorter in patients with higher ADA levels [13]. Diminished response to infliximab was also observed in ADA-positive patients with rheumatoid arthritis (RA) and ankylosing spondylitis [14–16]. Adalimumab, the completely human anti-TNF-α antibody, can also induce ADAs, which in some cases are associated with reduced efficacy in patients with RA [17, 18]. Antibodies to natalizumab, the humanized anti–very late activation antigen-4 (anti-VLA4), can cause loss of efficacy as well [19]. The precise mechanisms by which the efficacy of these therapeutic antibodies is diminished remain unclear without more detailed

ADA characterization. Some clinical observations suggest that binding of ADA to the drug increases its clearance. However, it is conceivable that some ADAs could neutralize efficacy by blocking a drug's complementarity-determining regions (CDRs) or adjacent epitopes. Monitoring such neutralizing ADAs with appropriate assays might be necessary if there is a loss of efficacy even in the absence of clearing antibodies. With the more widespread and more complex use of therapeutic antibodies, new types of risks have emerged. For example, RA patients who developed ADAs during treatment with infliximab more frequently developed ADAs to adalimumab in a subsequent "switch-over" treatment with adalimumab [20]. Furthermore, ADA responses may be influenced by allotypic differences between the therapeutic antibody and a patient's own immunoglobulins [21]. For example, the ADA response against adalimumab is higher in a Japanese population than in Caucasians due to allotypic differences [22]. These and other observations [23–25] suggest that genetic factors may play a role in the ADA responses, which should not be overlooked during clinical development. Nevertheless, clinical experience so far supports the view that humanized or human therapeutic antibodies generally fall into the category of biopharmaceuticals with lower to moderate risk.

Multicomponent biopharmaceuticals may add another level of risk because even the antibodies directed to the nonactive component of a drug could result in adverse clinical outcomes. For example, in patients treated with pegylated asparaginase, antibodies to polyethylene glycol (PEG) have been associated with changes in pharmacokinetics (PK) and loss of product efficacy [26]. Anti-PEG antibodies also have been associated with changes in PK and infusion reactions even in the absence of antibodies to the active protein component. It is, therefore, important to develop and implement separate assays specific to different components of a multicomponent biopharmaceutical.

Special consideration should be given to biopharmaceuticals that are used in treatment of genetic deficiencies such as lysosomal storage diseases or hemophilia. Given the inherent lack of immune tolerance toward missing proteins in these conditions, the high frequency of ADAs (80–100%) directed against the replacement biopharmaceuticals is not surprising. Obviously, ADAs cannot cross-react with missing endogenous counterparts and the danger of autoimmune syndromes does not exist. However, because of the serious nature of the aforementioned diseases, the loss of efficacy due to ADAs could have adverse clinical consequences. Monitoring of total and neutralizing ADAs using appropriate assays is highly recommended because such data can be useful in adjusting the therapeutic regimen if necessary. For example, in the case of mucopolysaccharidosis VI (MPS VI), recombinant human arylsulfatase B (Naglazyme, BioMarin, Novato, CA) is used to diminish lysosomal accumulation of dermatan sulfate. The strategy for monitoring ADAs in this treatment included total as well as neutralizing ADA assays designed on the basis of Naglazyme's two-step mechanism of action. One assay is used to detect ADA-mediated inhibition of Nagalazyme's binding to its mannose-6-phosphate

receptor and the other to determine potential inhibitory effect on Naglazyme's catabolic activity [27]. The data available to date from the Naglazyme Phase IV Clinical Surveillance Program (NCT00214773) show a high incidence of seroconversion (92%). However, no significant correlation has been established between antibody levels and changes in urinary glycosaminoglycan (GAG), used as a pharmacodynamic efficacy marker [28]. One explanation of this observation could be that a sufficient quantity of free Naglazyme can be taken up by cells even in the presence of circulating ADAs and that the internalized drug does reach the lysosomal compartment and degrades accumulated GAG. Another explanation could be that the drug–ADA complexes dissociate within the lysosomal compartment due to the low pH and/or proteolytic degradation of antibodies, allowing the enzyme to exert its natural function. Similarly, preservation of drug efficacy despite the high incidence of seroconversion has been observed in other storage diseases treated with replacement enzymes [29, 30]. In contrast, those replacement proteins that act outside cells, such as recombinant factors VIII and IX, are much more vulnerable to inhibitory effects of ADAs [31, 32].

1.4. DRUG–ADA COMPLEXES

Formation of immune complexes between biopharmaceuticals and ADAs can pose significant risks as well. A case has been reported of a patient with Pompe disease who developed reversible nephrotic syndrome during prolonged, high-dose enzyme replacement therapy with recombinant human acid–alpha glucosidase (rhGAA; Myozyme). Due to the development of ADAs to rhGAA and concomitant clinical decline, escalating doses of rhGAA were administered as part of an experimental immune tolerance regimen. Histological evaluation of kidney tissue revealed glomerular deposition of immune complexes containing rhGAA and IgG ADAs in a pattern of membranous nephropathy [33].

Nephrotic syndrome also has been observed in patients with hemophilia B undergoing immune tolerance therapy to eliminate ADAs directed against recombinant factor IX. Although the incidence of ADAs in patients with hemophilia B is generally lower (30–40%), high levels of ADA and ongoing high-dose drug treatment can lead to the reversible nephrotic syndrome [34–36]. Therefore, it appears that the presence of high levels of both drug and ADAs increases the risk of immune complex formation and that kidneys are particularly vulnerable to deposition of immune complexes. It was also found that antibody responses to rhGAA depend on the presence or absence of cross-reactive immunological material (CRIM). Patients with deleterious GAA mutations who are completely unable to form native enzyme are CRIM-negative, whereas patients with some residual, functioning or nonfunctioning enzyme present are CRIM positive. Kishnani and colleagues showed that IgG antibodies to rhGAA developed earlier and titers were higher and more

sustained in the CRIM-negative patients. CRIM-negative status predicted poorer clinical outcomes and reduced overall survival in infants with Pompe disease who were treated with rhGAA [37]. The effect of CRIM status on therapeutic outcome appears to be mediated by antibody responses to the exogenous protein.

All therapeutic proteins pose the risk of IgE ADA response that could lead to a variety of allergic reactions. Anaphylactic shock is certainly the most serious reaction and can be life threatening if not immediately treated, usually with antihistamines, epinephrine, and cortisone. It is prudent to develop and implement assays such as drug-specific IgE and serum tryptase whenever clinical symptoms indicate potential allergic reactions. Detection of drug-specific IgE provides the rationale and justifies antihistamine pretreatment in subsequent drug administrations.

1.5. CONCLUSION

In conclusion, a risk-based strategy (see Fig. 1.1) should include comprehensive evaluation of the molecular characteristics of the biopharmaceutical, its target and mechanism of action, and therapeutic indication(s) as well as the intended patient population. In addition, route of administration, formulation,

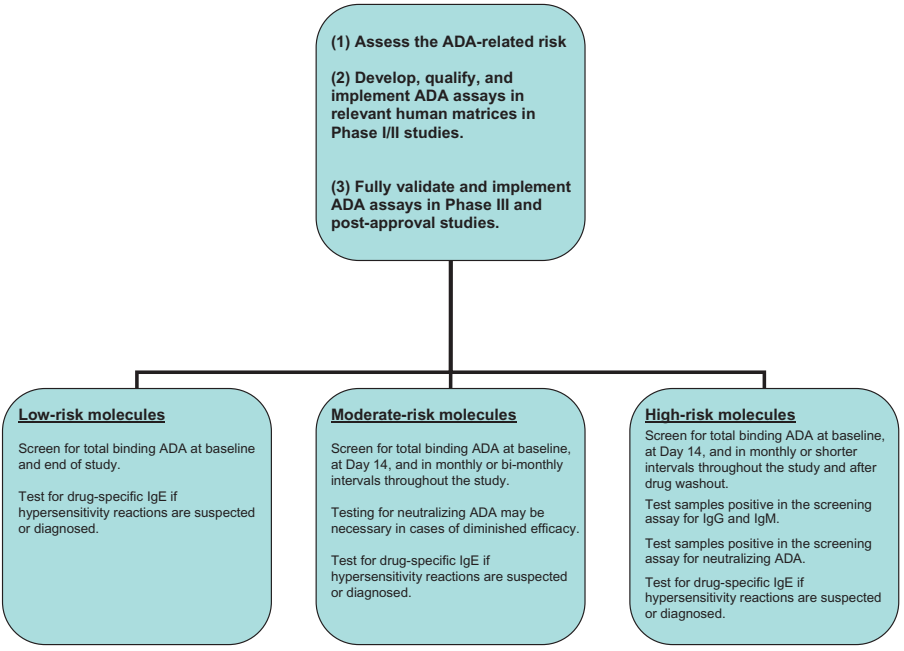
(1) Assess the ADA-related risk
(2) Develop, qualify, and implement ADA assays in relevant human matrices in Phase I/II studies.
(3) Fully validate and implement ADA assays in Phase III and post-approval studies.
Low-risk molecules
Screen for total binding ADA at baseline and end of study.
Test for drug-specific IgE if hypersensitivity reactions are suspected or diagnosed.
Moderate-risk molecules
Screen for total binding ADA at baseline, at Day 14, and in monthly or bi-monthly intervals throughout the study.
Testing for neutralizing ADA may be necessary in cases of diminished efficacy.
Test for drug-specific IgE if hypersensitivity reactions are suspected or diagnosed.
High-risk molecules
Screen for total binding ADA at baseline, at Day 14, and in monthly or shorter intervals throughout the study and after drug washout.
Test samples positive in the screening assay for IgG and IgM.
Test samples positive in the screening assay for neutralizing ADA.
Test for drug-specific IgE if hypersensitivity reactions are suspected or diagnosed.

Fig. 1.1 Flow diagram of recommended risk-based strategy for monitoring antibody responses to biopharmaceuticals during clinical development and after approval.

frequency of administration, dose levels, and concomitant immunomodulatory medications should be taken into account. Because of its complexity, the risk assessment should be carried out in a collaborative fashion between clinicians, toxicologists, pharmacokineticists, and assay experts. Timely consultations with regulatory agencies and/or clinical safety monitoring boards may be necessary as well. The identification of the risk level will affect the antibody testing scheme in terms of timing and frequency of sampling, neutralizing activity assessment, and qualitative, semiquantitative, or quantitative measurement, as well as isotype and affinity characterization. The greater the assessed risk, the more extensive and more frequent the ADA testing and characterization, along with cautious dosing regimens, that should be applied. It should be kept in mind that risk-based antibody monitoring will not make any biopharmaceutical product less immunogenic than it really is, but it will certainly help to minimize or even avoid antibody-related adverse effects. As the use and complexity of biopharmaceuticals have evolved, the implementation of a risk-based strategy has become increasingly important. An overall outline of risk-based strategy for ADA monitoring during clinical development is illustrated in Figure 1.1.

REFERENCES

1. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
2. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
3. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T., Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–1281.
4. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
5. Koren, E. (2002). From characterization of antibodies to prediction of immunogenicity. *Dev Biol*, *109*, 87–95.
6. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure

red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.

7. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
8. Ross, C., Clemmesen, K.M., Svenson, M., Sørensen, P.S., Koch-Henriksen, N., Skovgaard, G.L., Bendtzen, K. (2000). Immunogenicity of interferon-beta in multiple sclerosis patients: influence of preparation, dosage, dose frequency, and route of administration. Danish Multiple Sclerosis Study Group. *Ann Neurol*, *48*, 706–712.
9. Koren, E. (2003). Strategies for the preclinical and clinical characterization of immunogenicity. State of the art methods for the characterization of biological products and assessment of comparability. International Association for Biologics, National Institutes of Health, Bethesda, MD, June 10–13.
10. Zipkin, I. (1998). Amgen lays MGDF to rest. *BioCentury*, *6*, A8.
11. Bennett, C.L., Cournoyer, D., Carson, K.R., Rossert, J., Luminari, S., Evens, A.M., Locatelli, F., Belknap, S.M., McKoy, J.M., Lyons, E.A., Kim, B., Sharma, R., Costello, S., Toffelmire, E.B., Wells, G.A., Messner, H.A., Yarnold, P.R., Trifilio, S.M., Raisch, D.W., Kuzel, T.M., Nissenson, A., Lim, L.C., Tallman, M.S., Casadevall, N. (2005). Long-term outcome of individuals with pure red cell aplasia and antierythropoietin antibodies in patients treated with recombinant epoetin: a follow-up report from the Research on Adverse Drug Events and Reports (RADAR) Project. *Blood*, *106*, 3343–3347.
12. Swanson, S.J., Ferbas, J., Mayeux, P., Casadevall, N. (2004). Evaluation of methods to detect and characterize antibodies against recombinant human erythropoietin. *Nephron Clin Pract*, *96*, c88–c95.
13. Baert, F., Noman, M., Vermeire, S., Van Assche, G., D' Haens, G., Carbonez, A., Rutgeerts, P. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med*, *348*, 601–608.
14. Wolbink, G.J., Vis, M., Lems, W., Voskuyl, A.E., de Groot, E., Nurmohamed, M.T., Stapel, S., Tak, P.P., Aarden, L., Dijkmans, B. (2006). Development of antiinfliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis. *Arthritis Rheum*, *54*, 711–715.
15. de Vries, M.K., Wolbink, G.J., Stapel, S.O., de Groot, E.R., Dijkmans, B.A., Aarden, L.A., van der Horst-Bruinsma, I.E. (2007). Inefficacy of infliximab in ankylosing spondylitis is correlated with antibody formation. *Ann Rheum Dis*, *66*, 133–134.
16. Radstake, T.R., Svenson, M., Eijsbouts, A.M., van den Hoogen, F.H., Enevold, C., van Riel, P.L., Bendtzen, K. (2009). Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. *Ann Rheum Dis*, *68*, 1739–1745.
17. Bender, N.K., Heilig, C.E., Dröll, B., Wohlgemuth, J., Armbruster, F.P., Heilig, B. (2007). Immunogenicity, efficacy and adverse events of adalimumab in RA patients. *Rheumatol Int*, *27*, 269–274.
18. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Stapel, S., Lems, W.F., Aarden, L., Dijkmans, B.A., Tak, P.P., Wolbink, G.J. (2007). Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis*, *66*, 921–926.

19. Calabresi, P.A., Giovannoni, G., Confavreux, C., Galetta, S.L., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., O'Connor, P.W., Phillips, J.T., Polman, C.H., Radue, E.W., Rudick, R.A., Stuart, W.H., Lublin, F.D., Wajgt, A., Weinstock-Guttman, B., Wynn, D.R., Lynn, F., Panzara, M.A., AFFIRM and SENTINEL Investigators. (2007). The incidence and significance of anti-natalizumab antibodies: results from AFFIRM and SENTINEL. *Neurology*, *69*, 1391–1403.
20. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Stapel, S.O., Lems, W.F., Aarden, L., Dijkmans, B.A., Tak, P.P., Wolbink, G.J. (2009). Anti-infliximab and anti-adalimumab antibodies in relation to response to adalimumab in infliximab switchers and anti-TNF naive patients: a cohort study. *Ann Rheum Dis*, Aug 13 [Epub ahead of print].
21. Magdelaine-Beuzelin, C., Vermeire, S., Goodall, M., Baert, F., Noman, M., Assche, G.V., Ohresser, M., Degenne, D., Dugoujon, J.M., Jefferis, R., Rutgeerts, P., Lefranc, M.P., Watier, H. (2009). IgG1 heavy chain–coding gene polymorphism (G1m allotypes) and development of antibodies-to-infliximab. *Pharmacogenet Genomics*, *19*, 383–387.
22. Hayakawa,T., Ishii, A. (2011). Japanese regulatory perspective on immunogenicity. In *Detection and Quantification of Antibodies to Biopharmaceuticals: Practical and Applied Considerations*. Tovey MG, ed. John Wiley and Sons, New York, chap. 4 (this volume).
23. Koren, E., De Groot, A.S., Jawa, V., Beck, K.D., Boone, T., Rivera, D., Li, L., Mytych, D., Koscec, M., Weeraratne, D., Swanson, S., Martin, W. (2007). Clinical validation of the "in silico" prediction of immunogenicity of a human recombinant therapeutic protein. *Clin Immunol*, *124*, 26–32.
24. Barbosa, M.D., Vielmetter, J., Chu, S., Smith, D.D., Jacinto, J. (2006). Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity. *Clin Immunol*, *118*, 42–50.
25. Hoffmann, S., Cepok, S., Grummel, V., Lehmann-Horn, K., Hackermüller, J., Stadler, P.F., Hartung, H.P., Berthele, A., Deisenhammer, F., Wassmuth, R., Hemmer, B. (2008). HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of antibodies against interferon-beta therapy in multiple sclerosis. *Am J Hum Genet*, *83*, 219–227.
26. Armstrong, J.K., Hempel, G., Koling, S., Chan, L.S., Fisher, T., Meiselman, H.J., Garratty, G. (2007). Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*, *110*, 103–111.
27. White, J.T., Argento Martell, L., Prince, W.S., Boyer, R., Crockett, L., Cox, C., Van Tuyl, A., Aguilera, A., Foehr, E. (2008). Comparison of neutralizing antibody assays for receptor binding and enzyme activity of the enzyme replacement therapeutic Naglazyme (galsulfase). *AAPS J*, *10*, 439–449.
28. Harmatz, P., Giugliani, R., Schwartz, I.V., Guffon, N., Teles, E.L., Miranda, M.C., Wraith, J.E., Beck, M., Arash, L., Scarpa, M., Ketteridge, D., Hopwood, J.J., Plecko, B., Steiner, R., Whitley C.B., Kaplan, P., Yu, Z.F., Swiedler, S.J., Decker, C.; MPS VI Study Group. (2008). Long-term follow-up of endurance and safety outcomes during enzyme replacement therapy for mucopolysaccharidosis VI: final results of three clinical studies of recombinant human *N*-acetylgalactosamine 4-sulfatase. *Mol Genet Metab*, *94*, 469–475.

29. Germain, D.P., Waldek, S., Banikazemi, M., Bushinsky, D.A., Charrow, J., Desnick, R.J., Lee, P., Loew, T., Vedder, A.C., Abichandani, R., Wilcox, W.R., Guffon, N. (2007). Sustained, long-term renal stabilization after 54 months of agalsidase beta therapy in patients with Fabry disease. *J Am Soc Nephrol*, *18*, 1547–1557.
30. Wraith, J.E., Beck, M., Lane, R., van der Ploeg, A., Shapiro, E., Xue, Y., Kakkis, E.D., Guffon, N. (2007). Enzyme replacement therapy in patients who have mucopolysaccharidosis I and are younger than 5 years: results of a multinational study of recombinant human alpha-L-iduronidase (laronidase). *Pediatrics*, *120*, e37–e46.
31. Gouw, S.C., van der Bom, J.G., Auerswald, G., Ettinghausen, C.E., Tedgård, U., van den Berg, H.M. (2007). Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. *Blood*, *109*, 4693–4697.
32. Chitlur, M., Warrier, I., Rajpurkar, M., Lusher, J.M. (2009). Inhibitors in factor IX deficiency, a report of the ISTH-SSC international FIX inhibitor registry (1997–2006). *Haemophilia*, *15*, 1027–1031.
33. Hunley, T.E., Corzo, D., Dudek, M., Kishnani, P., Amalfitano, A., Chen, Y.T., Richards, S.M., Phillips, J.A. 3rd, Fogo, A.B., Tiller, G.E. (2004). Nephrotic syndrome complicating alpha-glucosidase replacement therapy for Pompe disease. *Pediatrics*, *114*, e532–e535.
34. Cross, D.C., Van Der Berg, H.M. (2007). Cyclosporin A can achieve immune tolerance in a patient with severe haemophilia B and refractory inhibitors. *Haemophilia*, *13*, 111–114.
35. DiMichele, D.M., Kroner, B.L. (2002). The North American Immune Tolerance Registry: practices, outcomes, outcome predictors: North American Immune Tolerance Study Group. *Thromb Haemost*, *87*, 52–57.
36. DiMichele, D.M. (2006). Immune tolerance: critical issues of factor dose, purity and treatment complications. *Haemophilia* (Suppl. 6), 81–85.
37. Kishnani, P.S., Goldenberg, P.C., DeArmey, S.L., Heller, J., Benjamin, D., Young, S., Bali, D., Smith, S.A., Li, J.S., Mandel, H., Koeberl, D., Rosenberg, A., Chen, Y-T. (2010). Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab*, *99*, 26–33.

PART II

REGULATORY REQUIREMENTS

2

IMMUNOGENICITY OF THERAPEUTIC PROTEINS: A REGULATORY PERSPECTIVE[1]

Susan Kirshner

2.1. INTRODUCTION

Protein products administered to people for therapeutic or diagnostic purposes may induce immune responses against themselves in the treated subjects [1]. As is discussed in more detail later in this chapter, immune responses that are specific to an administered protein may negatively impact product safety and efficacy. The laws and regulations that apply to protein therapeutics, namely, the Public Health Service Act of 1944 (Section 351) and the Federal Food, Drug, and Cosmetic Act (and as amended as part of the Kefauver Harris Amendments for Efficacy 1961 and the Food and Drug Administration Modernization Act of 1997), as well as the Code of Federal Regulations Title 21 [2], stipulate that the safety and efficacy of a product must be ensured. Therefore, assessing the immunogenicity of administered proteins and its impact on safety and efficacy is a critical part of product development and postmarketing surveillance.

The current state of the art does not allow for accurate prediction of who will develop immune responses and at what point during treatment the responses will develop. However, several factors that increase the likelihood

[1]Disclaimer: This chapter reflects the current thinking and experience of the author and should not be used in lieu of regulations, published FDA guidance, or direct discussions with the FDA.

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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of generating an immune response have been identified. This chapter explores the risks to patients from immune responses to protein therapeutics[2] and discusses current approaches to assessing such risks. This chapter does not focus on the development and validation of assays to evaluate antibodies to therapeutic proteins [also called antitherapeutic antibodies (ATAs)], since this has been covered in several white papers and in a recently published Draft Guidance by the US Food and Drug Administration (FDA) [3–6]. In addition, this chapter also does not address nonclinical immunogenicity studies or immunogenicity assessment for the approval of biosimilar products.

2.2. RISKS OF ANTITHERAPEUTIC IMMUNE RESPONSES

Immune responses to protein therapeutics pose a variety of risks to patients [7]. While in most cases little to no clinical impact of ATAs is observed, immune responses can have life-threatening consequences and/or modify the pharmacokinetics and pharmacodynamics of the therapeutic. Life-threatening type I hypersensitivity (anaphylactic) responses mediated by immunoglobulin IgE or IgG antibodies may be elicited by widely used products such as insulin [8], IL-2 diphtheria toxin [9], and glucocerebrosidase [10] among others. However, in general, anaphylactic responses to therapeutic proteins are rare.

Serious and life-threatening immune responses can occur when ATAs cross-react with and neutralize the endogenous protein counterpart of a protein therapeutic. For example, erythropoietin (EPO) is a nonredundant hematopoietic factor that is required for the development of erythrocytes. Antibodies to recombinant human EPO (rhEPO) that neutralize the product as well as endogenous EPO have resulted in patients developing pure red cell aplasia (PRCA). Although the incidence of PRCA is generally very low (1 in 10,000 patient-years), two incidents of increased PRCA frequency associated with formulation changes, container closure changes, and cold-chain problems were reported in Europe and Asia [11, 12]. Similarly, antibodies to pegylated megakaryocyte growth and development factor (PEG-MGDF) that cross-reacted with endogenous MGDF resulted in severe thrombocytopenia in 4% of treated healthy volunteers [13–15].

Antitherapeutic antibodies can also reduce the efficacy of life-saving therapies. Thus, persistent neutralizing antibodies (NAbs) to replacement therapies for metabolic disorders such as Pompe's, Fabry's, and Gaucher's diseases can result in a plateau in clinical improvement or disease progression and death [16, 17]. Similarly, inhibitory antibodies to factor VIII greatly complicate treatment of factor VIII–deficient hemophiliacs [18, 19].

Less drastic consequences of ATAs include impacts on product pharmacokinetics. ATAs can enhance clearance or, more uncommonly, diminish clearance. Persistent anti-natalizumab antibodies increased drug clearance, reduced

[2]For the purposes of this chapter the term "therapeutic" applies to proteins that are administered for both diagnostic and therapeutic purposes.

drug trough levels, and led to loss of clinical efficacy [20]. Antibodies to human growth hormone (hGH) have been reported to extend product half-life, although they did not appear to alter growth velocity in children [21]. Anti-insulin antibodies may delay the insulin response in some patients [8]. In addition to altering pharmacokinetics, ATAs may also alter pharmacodynamics by altering product targeting. Thus, ATAs to therapeutic replacement enzymes have caused enhanced uptake of product in plasma, lung, and kidney relative to target tissues, in rat studies with *N*-acetylgalactosamine 4-sulfatase [which is deficient in mucopolysaccharidosis (MPS) VI patients] and α-L-iduronidase (deficient in MPS I patients) [17].

Of theoretical concern is the impact of ATAs on fetal development since IgG antibodies can cross the placental barrier. Bone morphogenetic proteins (BMP) are critically involved in fetal development as well as in bone formation and repair and potentially in other functions in adults [22, 23]. The incidence of anti-BMP-2 antibodies is generally less than 1% in BMP-2-treated adults, whereas the incidence of antibodies to osteogenic protein-1 [OP-1 (BMP-7)] is very high (>90%) in OP-1-treated adults. The presence of such antibodies raises concerns regarding fetal development in antibody-positive mothers. This is true even in cases where antibody levels have reverted to baseline, since fetal expression of these proteins may restimulate the antibody response [10].

2.3. DEVELOPMENT OF ANTITHERAPEUTIC IMMUNE RESPONSES

Immune responses may be categorized as adaptive or innate, with the understanding that there are interactions and cross talk between these types of responses. Innate immune responses include inflammation and complement activation, and they facilitate adaptive immune responses. Adaptive immune responses are typified by the establishment of memory T and/or B cells that allow for more rapid responses to an antigen during a subsequent challenge. Immunological tolerance can be defined as "a physiologic state in which the immune system does not react destructively against components of an organism that harbors it or against antigens that are introduced to it" [24]. This section focuses on product quality attributes that have been implicated in breaking tolerance and/or increasing the likelihood of an antitherapeutic immune response.

As noted earlier, we cannot accurately predict the immunogenicity profile of therapeutic protein products. However, a number of factors have been identified that can impact product immunogenicity, including the following: (1) the source of the therapeutic (foreign or endogenous protein); (2) product-specific attributes; (3) patient-specific attributes; and (4) how the therapeutic is dosed and administered. Each of these factors will be discussed in more detail in the following subsections.

2.3.1. Product Derivation

Hosts are generally tolerant of self-proteins and intolerant of foreign proteins. Therefore, therapeutic proteins that are homologous to endogenous human proteins are generally less immunogenic in patients than foreign proteins (e.g., bacterial proteins). This lower immunogenicity may be expressed as a lower incidence of anti-drug antibody formation, a less robust immune response (lower titer and/or transient), or both.

2.3.2. Product-Specific Factors

Product-specific factors include structural and functional characteristics of the product, such as molecular structure, immunomodulatory properties, impurities, and formulation. Aspects of molecular structure that may impact immunogenicity include the presence of aggregates, novel epitopes, degradation variants (e.g., oxidation, deamidation), and glycosylation variants. Evidence for this is discussed later in this section. Discussion of the management of these factors is deferred to Section 2.3.5, on the assessment and management of antitherapeutic immune responses.

Aggregation of proteins has long been known to increase protein immunogenicity. Although the mechanisms for this are not fully understood, it has been posited that aggregated proteins resemble repeated protein arrays such as those found on pathogens (e.g., viral protein coats) to which the immune system is predisposed to react [25]. See References 25 and 26 for more comprehensive discussions regarding the immunogenicity of protein aggregates.

Clinically, aggregates of protein therapeutics have been associated with immune-mediated adverse events. For example, early clinical preparations of human growth hormone (hGH) derived from human pituitary glands were highly aggregated (40–70%) and were immunogenic in approximately 50% of children who took them. Changes in the manufacturing process reduced the aggregates in the product. Although the incidence of anti-hGH antibodies remained high, anti-hGH antibodies in patients treated with higher levels of monomeric hGH tended to be transient, whereas anti-hGH antibodies were persistent in patients treated with more highly aggregated hGH [27]. Similarly, in the 1950s and early 1960s severe hypersensitivity reactions were observed in patients treated with aggregated intravenous immunoglobulin (IVIG). Anti-IVIG antibody responses in patients appeared to be specific for a normally cryptic epitope that is exposed on aggregated IVIG [28–30]. See Wang et al. [31] for a more complete discussion on protein aggregates.

Therapeutics that replicate endogenous human proteins may contain novel epitopes generated through a variety of pathways. Amino acid substitutions have been used to promote protein stability or solubility. Alternatively, naturally variant proteins may be used. A single amino acid change can modify the immunogenicity of a protein. For example, diabetic patients have been treated with recombinant human or naturally derived porcine and bovine insulins.

Porcine and human insulin differ by a single amino acid, whereas bovine and human insulins differ by two amino acids. Consistent with this, human insulin is less immunogenic than porcine insulin and porcine insulin is less immunogenic than bovine insulin in diabetic patients [7].

It should be noted that amino acid substitutions in a therapeutic protein may reduce product immunogenicity. Induction of an adaptive immune response to a therapeutic protein requires the activation of antigen-specific T and B lymphocytes. Computational, *in vitro*, and *in silico* methods have been developed to attempt to predict T- and B-cell epitopes on therapeutic proteins. However, due to the complexity of immune responses in humans and the current lack of knowledge about many aspects that influence immunogenicity, these methods cannot yet be relied upon as stand-alone tools for predicting immunogenicity. See References 32–34 for more complete discussions of this topic.

Novel epitopes can be generated in the joining regions of fusion proteins even if both are of human origin, so that the proteins appear as foreign and elicit an immune response. Finally, amino acid residues may be altered (e.g., by the addition of chemical moieties) to improve protein stability. Such changes to a protein that is homologous to an endogenous human protein can make it appear foreign to the immune system, thereby increasing immunogenicity.

Of concern is the possibility that, once immune responses are initiated against altered human molecules, the epitope specificity of the immune response can spread to portions of the molecule that are homologous and can potentially cross-react with the endogenous human counterpart. Consistent with this Brand et al. [35] reported that serum samples from three hepatitis C patients who developed NAbs to the interferon (IFN) subtype IFN-α2a also neutralized all the native human IFN-α subtypes found in lymphoblastoid IFN.

There is evidence that glycosylation changes can alter the immunogenic potential of proteins and peptides through a variety of mechanisms, including but not limited to exposing cryptic T- and B-cell epitopes in the protein, altering epitope processing and presentation by antigen-presenting cells, and exposing or introducing novel carbohydrate epitopes. It should be noted that the presence of complex carbohydrates is hypothesized to reduce immunogenicity, as complex carbohydrates are characteristic of higher eukaryotes and do not appear to be easily recognized by αβ T cells [36, 37].

Antibodies to cryptic epitopes have been observed in the clinic. For example, antibodies to recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) produced in yeast or *Escherichia coli* recognize a site on the protein backbone that is shielded in endogenous GM-CSF by an O-linked glycan [38]. Similarly, the type II collagen derived arthritogenic, major histocompatibility complex (MHC) class II restricted epitope in rheumatoid arthritis is a glycopeptide that is underglycosylated in rheumatoid arthritis patients [39].

Antigen processing and presentation can be influenced by N- and O- linked glycans that mask enzymatic cleavage sites [40]. Antigen presentation may

also be impacted by carbohydrate moieties sterically hindering peptide binding in the MHC groove. Thus MHC class I molecules have been found to present peptides with O-linked but not N-linked glycans [36, 40]. However, MHC class II molecules have been shown to present peptides with N-linked glycans [36].

Changes in carbohydrate moieties may cause a molecule to appear foreign to the immune system. Innate immune responses to malformed N-glycans that may mimic N-glycans of lower eukaryotes have been shown to cause tissue damage in an animal model of systemic lupus erythematosis [37, 41]. IgE-mediated anaphylactic responses to cetuximab in treated human patients were shown to be specific for the galactose-α-1,3-galactose carbohydrate moiety on the Fc portion of the monoclonal antibody [42]. Interestingly, a number of patients had pre-exisiting antibodies that cross-reacted with cetuximab. Environmental factors are hypothesized to contribute to the anti-cetuximab response as anaphylaxis was limited to specific geographic regions.

Although addition of polyethylene glycol (PEG) to proteins appears to reduce product immunogenicity for many proteins, it has more recently been appreciated that patients may develop antibodies to PEG, with adverse clinical impact. Anti-PEG asparaginase antibodies that abrogate product efficacy, presumably by increasing product clearance, have been observed in the clinic [43]. In a different example, the presence of anti-PEG antibodies was correlated with infusion reactions that worsened as anti-PEG antibody titers increased. Furthermore, anti-PEG antibodies to one pegylated therapeutic protein have been found to cross-react with unrelated pegylated products, raising the possibility that patients who have anti-PEG antibodies will be refractory to treatment with other pegylated products.

Many protein therapeutics are designed to modulate immune responses. Immunosuppressive therapies have been developed to treat autoimmune and inflammatory diseases, whereas immunostimulatory therapies have been used to boost antitumor responses in oncologic diseases. It has been hypothesized that the development of anti-drug antibodies may be suppressed in patients taking intrinsically immunosuppressive therapies and increased in patients taking intrinsically immunostimulatory therapies. However, such has not been found to be the case for many products, due to confounding factors such as disease state and concomitant medications. In addition, many of the immunomodulatory proteins have immunotoxicities [44–47] that complicate data interpretation.

Process-related impurities, particularly host-cell proteins and DNA, can increase product immunogenicity. Wadhwa et al. [48] reported that immunogenicity to GM-CSF was increased for a product containing *E. coli*–derived protein contaminants when compared with a second *E. coli*–derived GM-CSF product that did not contain the contaminants. Antibodies to two *E. coli* proteins were also detected in the serum of patients treated with the contaminant-containing product. Similarly, immune responses including hypersensitivity responses have been seen with yeast host-cell component contaminants in yeast-derived biotechnology products.

Nonbiological impurities from a variety of sources may also impact immunogenicity, primarily by nucleating protein aggregation. These include silicone oil droplets [49], glass chips [50], metal shavings [51], and metal derivatives (such as tungsten oxides) [52].

Product formulation may impact product immunogenicity primarily by altering protein stability [53]. Moreover, reducing sugars present in formulations for cryoprotection can directly glycate proteins, thereby changing their carbohydrate profile [54]. In addition a formulation with human serum albumin (HSA) has been associated with the formation of heteroaggregates between the HSA and IFN-α2a [55]. Removal of HSA and reduced temperature resulted in less aggregate formation and reduced immunogenicity [56]. This is not surprising, as HSA is known to form noncovalent associations with proteins, and this may be relevant to other HSA-formulated products.

Container closure systems can also contribute to product immunogenicity. The primary purpose of container closures is to act as a barrier between the drug and the external environment. Insufficient protection from gas exchange, changes in moisture level, and light can all negatively impact product quality [57]. In addition, contaminants that leach from the container closure can negatively impact product quality. For example, tungsten oxides deposited in some syringes as a result of the syringe manufacturing process oxidized an IFN-β preparation, which led to increased formation of IFN-β aggregates [58]. A variety of metals, minerals, and vulcanizing agents can leach from either the glass or rubber stopper components of container closures. These may impact product quality directly, as just noted, or indirectly as evidenced by the activation of a metalloprotease contaminant of a drug product that resulted in product degradation [58]. Of interest is the potential for contaminants to directly activate the immune system and act as adjuvants. This has been hypothesized as a mechanism for the increased incidence of PRCA in Europe in 1998–2002 [59]. More recently it has been found that nickel allergy may be due, at least in part, to the ability of nickel to directly activate immune cells via the Toll-like receptor 4 [60]. Thus the presence of metals that leach from containers or other product contact equipment may impact product immunogenicity. See Sharma [57] for a more complete discussion of the impact of container closures on immunogenicity.

Product handling can impact the stability and therefore the immunogenicity of therapeutic proteins. For example, IFN-α2a immunogenicity was correlated with storage temperature as well as formulation [55]. Thus, IFN-α2a stored at 30°C was more immunogenic than when stored at 4°C. IFN-α2a immunogenicity was exacerbated in HSA-containing formulations under these conditions. This was attributed to an increase in aggregates as well as an increase in oxidized species, which may ultimately have contributed to aggregate formation. Aggregates were found in both the HSA-containing as well as HSA-free formulations. However, HSA–IFN-α2a heteroaggregates in addition to self-aggregates were found in the HSA-containing formulation. Formulation modification, better control over storage conditions, and better product purity were implemented to reduce immunogenicity [55, 56].

Oxidation is a major degradation pathway for the monoclonal antibody muromonab-CD3 (Orthoclone OKT3, Centocor Ortho Biotech Products) and is associated with loss of potency [61]. Kroon et al. [61] found that oxygen levels in ambient air were sufficient to result in product oxidation and loss of potency after 9 months. Storing muromonab under a nitrogen overlay significantly improved product stability with regards to oxidation. Similarly agitation as well as temperature was found to increase the formation insulin degradation products [62]. This is significant because carrying insulin in a shirt pocket was found to be sufficient to increase product degradation.

2.3.3. Patient-Specific Attributes

A variety of patient-specific attributes can contribute to the development of anti-drug immunity. These include genetics, disease status, concomitant medications, age, and gender. It has long been established that there is a strong genetic component to immune responses. A critical component of the genetics of adaptive immune responses is the expression of major histocompatability molecule haplotype. Thus the human leukocyte antigen HLA DRB1*0701 has been implicated in susceptibility to anti-IFN-β antibody responses in treated multiple sclerosis (MS) patients [63]. Similarly, high levels of anti-insulin antibodies were associated with HLA DR4 expression in some studies, whereas low anti-insulin immune response was associated with HLA DR3 expression [64].

The immunogenicity of protein therapeutics designed to replace missing or defective self-proteins is impacted by the nature of the mutation in the gene coding for the factor. Thus the development of clinically relevant anti–factor VIII antibodies is less common in patients classified as mild. However, a subset of mild hemophilia A patients with missense mutations in regions of factor VIII that are commonly targeted by anti–factor VIII antibodies have a higher incidence of clinically relevant antibody development than patients with mutations outside these regions. It is hypothesized that in these cases the administered wild-type factor VIII is perceived as foreign by the immune system [65]. Hemophilia A patients with mutations that result in a lack of circulating factor VIII are more likely to develop clinically neutralizing anti–factor VIII antibodies upon treatment [65]. In all these cases it is hypothesized that the replacement protein is perceived as foreign by the host immune system [65].

The development of NAbs to replacement enzymes for the treatment of inborn errors of metabolism is associated with the severity of the genetic lesion. Thus in Gaucher's disease, only 13% of patients develop antibodies to glucocerebrosidase because nearly all patients with this disease are positive for cross-reactive immunologic material (CRIM). Thus only a rare patient with Gaucher's develops NAbs that can lead to loss of efficacy and disease progression. Similar findings were reported for CRIM-positive Pompe's infants on acid beta glucosidase replacement therapy. However, earlier, higher, and more

sustained ATAs were detected in CRIM-negative infants. These patients also had worse clinical outcome, with all CRIM-negative but only 19% of CRIM-positive patients deceased or invasively ventilated by 27.1 months [66].

The immune competency of patients also contributes to product immunogenicity. This can be impacted by age, gender, disease state, and concomitant medications. For example, the development of anti-PEG-MGDF antibodies is less prevalent in cancer patients, who tend to be immune suppressed, than in healthy individuals. Thus, approximately 0.6% of cancer patients developed NAbs to PEG-MGDF, whereas 4% of healthy platelet donors developed NAbs [13–15]. In some patients anti-PEG-MGDF NAbs cross-reacted with endogenous MGDF, resulting in devastating thrombocytopenia [14, 67]. Similarly, antibodies to IFN-β were not detected in patients treated for hairy cell leukemia, Kaposi's sarcoma, or condyloma acuminata [68], although 5–70% of patients treated for the autoimmune disease MS develop antibodies to IFN-β during treatment.

Concomitant medications can also influence product immunogenicity by impacting the immune competency of the patient. Thus, human-anti-chimera antibodies (HACAs) to adalimumab were reduced in rheumatoid arthritis patients concomitantly treated with the immunosuppressant methotrexate [69]. HACAs were also reduced in infliximab-treated patients with Crohn's disease who were co-treated with immunosuppressive agents [70]. Similarly, the prevalence of NAbs to IFN-β1b at 15 months was reduced from 26.8% to 12.1% by concomitant immunosuppressive therapy (methylprednisone) [71].

2.3.4. Dosing and Administration

The dosing and administration of therapeutic proteins can contribute to immunogenicity. Low-affinity and low-titer IgM responses are typical after a single exposure to antigen. Multiple antigen exposures result in the development of class-switched (IgG, IgA, IgE) higher affinity and frequently higher titer antibody responses. Therapeutic proteins that will be given only once are far less likely to generate ATAs, with the caveat that patients may previously have been naturally and/or unknowingly exposed to some proteins (e.g., through diet, infection). However, intermittent exposure, which typifies many dosing regimens, resembles prime–boost regimens that induce adaptive immune responses. Consistent with this, Schernthaner [8] reported that intermittent dosing of insulin appeared to lead to increased risk of severe insulin allergy, particularly if nonhuman insulin was used.

Duration of exposure may also impact immunogenicity. Antonelli et al. [72] reported a higher incidence of anti-IFN-α2a antibodies in patients receiving long-term treatment of chronic myelogenous leukemia (CML) or hairy cell leukemia (HCL) compared with patients treated for a shorter period. Antibodies to IFN-β-treated patients generally emerge during the first year of treatment, although they have been found to emerge for up to two years. The development of anti-IFN-β NAbs usually occurs after the appearance of

non-neutralizing antibodies, sometimes not until the third and fourth years of treatment [73].

Route of exposure can impact product immunogenicity. Thus oral, nasal, and intravenous (i.v.) routes of exposure are generally considered least likely to generate an immune response, followed by intramuscular (i.m.) administration. Subcutaneous (s.c.) administration is considered the most immunogenic, probably due to the high level of immune surveillance in the skin. Consistent with this, i.v. insulin is nonimmunogenic, whereas s.c. insulin can be highly immunogenic [8]. However, Bertolotto et al. [74] reported that the prevalence of NAbs was equivalent for the same preparation of IFN-β1a whether administered i.m. or s.c. [74].

2.3.5. A Regulatory Perspective on Assessment and Management of Antitherapeutic Immune Responses

There are several components to the assessment and management of product immunogenicity, including (1) the risk of developing clinically relevant ATAs, (2) the proposed immunogenicity assessment program, (3) assay development and validation (not discussed here), (4) control of product quality, and (5) approaches to clinical management.

2.4. IMMUNOGENICITY RISK

The FDA "Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins" [6] recommends a risk-based approach to immunogenicity assessment during product development. Although the Draft Guidance does not provide much detail about what should be included in a risk-based approach, we are most concerned with the development of antibodies that have clinical impact. The Draft Guidance does recommend that because assays to detect immunogenicity "are critical when immunogenicity poses a high-risk and real time data concerning patient responses are needed, the applicant should implement preliminary validated assays early (preclinical and phase 1)." It further states that "the risk to patients of mounting an immune response to product will vary with the product" [6].

For many products in early stages of development the risk of ATAs is unknown. Important information regarding the potential ramifications of ATAs neutralizing endogenous counterparts may be obtained from the literature, including animal models (e.g., knock-out or conditional knock-out models) or human diseases in which antibodies to the endogenous counterpart are thought to play a role. It is useful to include such information in investigational new drug (IND) applications when providing the rationale for the product's immunogenicity program. In addition, the FDA considers all the factors that are thought to impact product immunogenicity when evaluating the adequacy of proposed immunogenicity assessment programs. The FDA considers additional factors when evaluating immunogenicity assessment pro-

grams, including whether the proposed therapeutic addresses an unmet medical need or a life-threatening disease.

In general, for single-dose safety studies, the FDA allows applicants to bank samples for future testing. Assays used to test clinical samples always ought to be suitable for their intended purpose, since otherwise they will not provide reliable results. For low-risk products it is frequently acceptable to begin single-dose phase 1 studies with assays under development. It is expected that suitable assays will be in place for multidose or later phase studies. According to the FDA Draft Guidance fully validated assays should be used to test clinical samples from pivotal trials. For high-risk products, for example, when serious immungenicity-related adverse events have occurred with that product or similar products or when there are already similar products with acceptable safety records on the market, validated immunogenicity assays are generally requested prior to initiation of phase 1 studies.

2.4.1. Immunogenicity Assessment Programs

In the Draft Guidance [6] the FDA recommends a multitiered approach to testing patient samples. This is summarized in Figure 2.1. Depending on the assay format used, the screening assay should minimally detect IgM and IgG antibodies for nonmucosal routes of administration. For mucosal routes of administration the ability to detect IgA isotype ATAs is also recommended. In most cases it is not necessary to develop assays to detect ATAs of the IgE isotype unless type 1 hypersensitivity reactions have been observed.

The specificity of antibodies that test positive in the screening assay is confirmed, usually by competition with unlabeled drug using the same assay

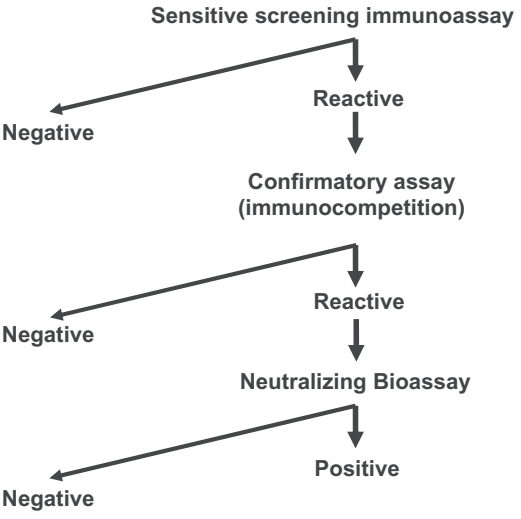

Fig. 2.1 Multitiered approach to testing patient samples.

format as the screening assay. Antibodies are frequently further characterized for titer and sometimes for isotype and epitope specificity. Determination of ATA titers in patients can be useful since adverse events and loss of efficacy are sometimes correlated with titer rather than the presence or absence of ATAs.

Antibodies that test positive in the binding and confirmatory assays are tested in a neutralizing-antibody assay. This is usually a cell-based bioassay; however, animal-based bioassays are also occasionally used. The purpose of the confirmatory assay is to assess whether there are antibodies present that interfere with the interaction between the therapeutic and the target. Determining the titers of NAbs may also be useful as these too have been correlated with adverse events and loss of efficacy.

For all assays test samples should be obtained at appropriate time points depending on the duration of the trial and the frequency of dosing. It is recommended that samples be obtained at trough levels of drug. However, for therapeutics with long half-life, serum drug levels may be sufficiently high to interfere with assay performance throughout the treatment phase of the clinical trial. Under such circumstances it is recommended that samples be taken after an appropriate washout period. Furthermore, it is recommended that patients who seroconvert during the course of the trial have ATAs monitored until they revert to baseline.

Other factors are also considered when evaluating the adequacy of immunogenicity assessment programs. For example, since the incidence and severity of antitherapeutic immune responses can vary between target populations, it is important that the incidence and clinical consequences be evaluated for each target population. Target populations may be framed by disease indication or by subpopulations in a disease indication. For example, immunogenicity is usually analyzed separately for adult and juvenile patients, even for the same indication.

The prevalence of ATA development varies greatly between products and indications. The occurrence of serious or life-threatening antitherapeutic responses may be too low to assess during clinical trials. Therefore it may be necessary to establish postapproval surveillance programs to continue to monitor for infrequent but clinically important ATA responses.

When a therapeutic protein has more than one functional domain, for example, a monoclonal antibody, assays to detect antibodies to the different domains are expected. Similarly, assays to detect PEG are frequently requested for pegylated products. In cases where there is high homology between the therapeutic protein and other endogenous proteins, assessing antisera cross-reactivity to those other proteins may be recommended.

2.4.2. Control of Product Quality

Control of product quality is vital for managing immunogenicity. To this end the FDA considers the assessment and control of aggregates to be crucial.

Aggregates less than 0.2 μm and greater than 10 μm are usually specified for parenteral therapeutic proteins. To date, aggregates between 0.2 and 10 μm have not been systematically assessed or specified [75]. However, the FDA is currently working to address this gap by requesting that manufacturers assess aggregates between 2 and 10 μm. The assessment of aggregates between 0.2 and 2 μm may also be monitored when suitable technologies become available. Other quality attributes that can impact product immunogenicity such as glycosylation, oxidation, and deamidation are usually specified.

It is important to thoroughly understand method capabilities when selecting the methods for assessing product quality. This can be achieved by confirming or assessing the results of the proposed routine test method using an alternative method. For example, size exclusion chromatography is the most frequently used release test for aggregates. During assay validation an orthogonal method such as analytical ultracentrifugation or field flow fractionation can be used to verify results. It is also advisable to study stressed samples to confirm that the assay can detect relevant degradation products. Similarly, since host-cell protein contaminants have been associated with increased immunogenicity, it is important to confirm that host-cell protein assays adequately detect the spectrum of host-cell proteins found in the drug product.

As degradation variants of therapeutic proteins may increase product immunogenicity, these must be monitored and controlled throughout shelf-life. More extensive testing at the proposed end-of-shelf-life may be useful to help guarantee product quality. An important parameter to test at the end-of-shelf-life is leachables and leachable-associated product variants, as these may accumulate over time.

Storage conditions can impact product quality. Thorough qualification of container closures, including the heterogeneity of the closures coming from a manufacturer, is important for controlling the impact of container closures on product quality. Results of forced degradation, accelerated stability, and agitation studies inform recommendations for storage and cold-chain management.

Because manufacturing changes can also impact product quality [76], it is critical to thoroughly evaluate the impact of manufacturing changes on product quality. If manufacturing changes have impacted critical quality attributes thought to contribute to product immunogenicity, human immunogenicity safety studies may be requested.

2.5. CLINICAL MANAGEMENT

The clinical management of antitherapeutic immune responses depends on many factors, including, but not limited to, observed clinical consequences of ATAs, disease state, alternative therapies, and potential management strategies. The clinical management of antitherapeutic immune responses should be

discussed and agreed upon with the appropriate clinical division at the FDA. This section highlights some of the strategies that have been used for the clinical management of ATAs.

Many strategies are available for management of hypersensitivity reactions. When there is a high risk of anaphylaxis the FDA usually requests that patients be treated in an environment where appropriate emergency care can be administered. In addition it is generally recommended that patients remain at the treatment center for observation for an appropriate period of time. In most cases patients are withdrawn from the study if they have had an anaphylactic response to the drug. Depending on the severity of the response, type 1 hypersensitivity responses are either adverse events or serious adverse events that should be reported to the FDA as IND safety reports per the Code of Federal Regulations Title 21 [2]. Strategies for controlling hypersensitivity reactions during infusions include, but are not limited to, reducing the infusion rate and/or prophylactic treatment with drugs such as antihistamines and/or immunosuppressants. In cases of severe reactions, infusions may be discontinued.

There are multiple strategies for dealing with loss of product efficacy due to the presence of ATAs. Frequently ATA responses are transient and disappear on their own with continued treatment. However, even the persistent presence of antibodies may not negatively impact treatment. For example, HCL patients with lower titers of antibody [below 400 international neutralizing units (INU)] remained responsive to therapy (IFN-α), whereas patients with more than 400 INU tended to become resistant to treatment. Steis et al. [77] reported the loss of anti-IFN-α2a antibodies with prolonged continuous IFN-α2a treatment [77]. Loss of antibodies to IFN-β over time has also been reported [72]. Kakavanos et al. [78] reported that antibodies to recombinant human iduronidase in MPS I (Hurler–Scheie's syndrome) patients disappeared over time. These antibodies were non-neutralizing but resulted in hypersensitivity reactions in some patients [78].

When alternative similar products are available, therapeutic efficacy may be regained by switching to an alternative product. Patients have been effectively switched to different insulin [64] or growth hormone [79, 80] products. Conflicting results have been obtained for IFN-β products. It has been reported that patients who develop clinically neutralizing antibody responses to IFN-β1b s.c. can be effectively switched to IFN-β1a i.m. [81]. However, Gneiss et al. [82] reported that switching from either IFN-β1a or IFN-β1b s.c. to IFN-β1b i.m. did not impact the course of NAb titers. Different methodologies were used in the two reports, which might explain the contradictory results. The Austrian study included 24 subjects who were followed for 12 months after randomization to either maintain or switch IFN-β treatments [82]. The open label extension of the phase III study of the Multiple Sclerosis Collaborative Research Group (IFN-β1a i.m.) included 23 patients who had previously been treated with IFN-β1b s.c. and were NAb positive at baseline. Six of those 20

patients (3 had dropped out) were NAb positive after 2 years, and 3 of 14 patients (6 had dropped out) were NAb positive at 5 years [81]. So it is possible that patients were not followed for a sufficiently long time in the Austrian switch study. Similarly, although it has been reported that some patients can be effectively switched between IFN-α products [72], the cross-reactivity of anti-IFN-α NAbs to native IFN-α subtypes suggests that product switching will not be effective in all patients. *In vitro* data indicate that antibodies to recombinant IFN-α can cross-react with other recombinant IFN-α products [83] or with native human IFN-α subtypes [35]. Similarly, recombinant IFN-β products may cross-react with other IFN-β products. However, patients taking recombinant EPO products who developed PRCA were refractory to treatment with other EPO products. Thus, the effectiveness of switching may need to be established on an individual basis.

An alternative approach that has been successfully implemented in some cases is the induction of immunological tolerance to the protein therapeutic. Tolerance to exogenous factor VIII has been established in more than 80% of selected hemophilia A patients, generally by exposure to high doses of factor VIII with concomitant immunosuppressant administration. High-dose immunoglobulin treatment and/or immunoadsorption on protein A are components of some tolerance protocols. Low and medium dose tolerance protocols have also been successfully attempted [18, 19]. A similar tolerance induction protocol was successfully implemented in a child with type 3 Gaucher's disease who developed NAbs to glucocerebrosidase that abrogated clinical efficacy [84].

In addition, plasmapherisis and immunoabsorption have been used to manage life-threatening complications from NAbs to factor VIII [85] and MGDF [13].

Finally, when the risks of the clinical consequences of product immunogenicity outweigh the potential benefits, then discontinuing treatment is an option.

2.6. SUMMARY

The Public Health Service Act of 1944 (Section 351) and the Federal Food, Drug, and Cosmetic Act (Section XX, and as amended as part of the Food and Drug Administration Modernization Act of 1997), as well as the Code of Federal Regulations Title 21, stipulate that the safety and efficacy of a product must be ensured. Since antitherapeutic immune responses have been found to have negative impacts on product safety and efficacy, it is crucial to evaluate these during product development and in the postmarketing environment. This chapter highlights our current knowledge and thinking about the risks of ATAs to safety and efficacy, the factors that contribute to the development of ATAs, and the strategies for controlling and managing ATAs.

REFERENCES

1. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
2. US Government. 2009. Code of Federal Regulations. Title 21, Food and Drugs.
3. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
4. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirshner, S., Moxness, M., Parish, T., Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–1281.
5. Gupta, A., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
6. Food and Drug Administration (FDA). (2009). Draft guidance for industry: assay development for immunogenicity testing of therapeutic proteins. CEDER. December 2009.
7. Rosenberg, A.S. (2003). Immunogenicity of biological therapeutics: a hierarchy of concerns. *Dev Biol Basal*, *112*, 15–21.
8. Schernthaner, G. (1993). Immunogenicity and allergenic potential of animal and human insulins. *Diabetes Care*, *16* (Suppl. 3), 155–165.
9. Foss, F.M., Bacha, P., Osann, K.E., Demierre, M.V., Bell, T., Kuzel, T. (2001). Biological correlates of acute hypersensitivity events with DAB(389)IL-2 (denileukin diftitox, ONTAK) in cutaneous T-cell lymphoma: decreased frequency and severity with steroid premedication. *Clin Lymphoma*, *1* (4), 298–302.
10. Rosenberg, M., Kingma, W., Fitzpatrick, M.A., Richards, S.M. (1999). Immunosurveillance of alglucerase enzyme therapy for Gaucher patients: induction of humoral tolerance in seroconverted patients after repeat administration. *Blood*, *93*, 2081–2088.
11. Sharma, B. (2007). Immunogenicity of therapeutic proteins. Part 1. Impact of product handling. *Biotech Adv*, *25*, 310–317.
12. Fotiou, F., Aravind, S., Wang, P-P., Nerapusee, O. (2009). Impact of illegal trade on the quality of epoetin alfa in Thailand. *Clin Ther*, *31* (2), 336–346.
13. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
14. Basser, R., O'Flaherty, E., Green, M., Edmonds, M., Nichol, J., Menchaca, D., Cohen. B., Begley, C.. (2002). *Development* of pancytopaenia with neutralizing antibodies

to thrombopoietin after multicycle chemotherapy supported by megakaryocyte growth and development factor. *Blood*, *99*, 2599–2602.

15. Kuter, D.J. (2009). Thrombopoietin and thrombopoietin mimetics in the treatment of thrombocytopenia. *Annu Rev Med*, *60*, 193–206.
16. Ponce, E., Moskovitz, J., Grabowski, G. (1997). Enzyme therapy in Gaucher disease type I: effect of neutralizing antibodies to acid-glucosidase. *Blood*, *90*, 43–48.
17. Brooks, D.A., Kakavanos, R., Hopwood, J.J. (2003). Significance of immune response to enzyme-replacement therapy for patients with lysosomal storage disorder. *Trends Mol Med*, *9* (10), 450–453.
18. Brackmann, H.H., Lenk, H., Scharrer, I., Auerswald, G., Kreuz, W. (1999). German recommendations for immune tolerance therapy in type A haemophiliacs with antibodies. *Haemophilia*, *5*, 203–206.
19. Hay, C.R., Baglin, T.P., Collins, P.W., Hill, F.G., Keeling, D.M. (2000). The diagnosis and management of factor VIII and IX inhibitors: a guideline from the UK Haemophilia Centre Doctors' Organization (UKHCDO). *Br J Haematol*, *111*, 78–90.
20. Calabresi, P.A., Giovannoni, G., Confavreux, C., Galetta, S.L., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D.H., O'Connor, P.W., Phillips, J.T., Polman, C.H., Radue, E.W., Rudick, R.A., Stuart, W.H., Lublin, F.D., Wajgt, A., Weinstock-Guttman, D.R., Lyn, F., Panzara, M.A. (2007). The incidence and significance of anti-natalizumab antibodies. *Neurology*, *69*, 1391–1403.
21. Pringle, P.H., Hindmarsh, P.C., Di Silvio, L., Teale, J.D., Kurtz, A.B., Brook, C.G. (1989). The measurement and effect of growth hormone in the presence of growth hormone–binding antibodies. *J Endocrinol. 121* (1), 193–199.
22. Chen, E., Zhao, M., Mundy, G.R. (2004). Bone morphogenic proteins. *Growth Factors*, *22* (4), 233–241.
23. Rosen, V. (2009). BMP2 signaling in bone development and repair. *Cytokine Growth Factor Rev*, *20*, 475–480.
24. Schwartz, R. (1999). Immunological tolerance. In *Fundamental Immunology*, 4th edition. Paul WE, ed. Lippencott-Raven Publishers, Philadelphia.
25. Rosenberg, A.S. (2006). Effects of protein aggregates: an immunologic perspective. *AAPS J*, *8* (3), E501–E507.
26. Filipe, V., Hawe, A., Schellekens, H., Jiskoot, W. (2010). Aggregation and immunogenicity of therapeutic proteins. In *Aggregation of Therapeutic Proteins*. Wang W, Roberts CJ, eds. John Wiley and Sons, Hoboken, NJ.
27. Moore, W.V., Leppert, P. (1980). Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J Clin Endocrinol Metab*, *51* (4), 691–697.
28. Barandun, S., Kistler, P., Jeunet, F., Isliker, H. (1962). Intravenous administration of human γ-globulin. *Vox Sang*, *7*, 157–174.
29. Ellis, E.F., Henney, C.S. (1969). Adverse reactions following administration of human gamma globulin. *J Allergy*, *43* (1), 45–54.
30. Gamble, C.N. (1966). The role of soluble aggregates in the primary immune response of mice to human gamma globulin. *Int Arch Allergy Appl Immunol*, *30* (5), 446–55.
31. Wang, W., Nema, S., Teagarden, D. (2010). Protein aggregation—pathways and influencing factors. *Int J Pharm*, *390*, 89–99.

32. Bryson, C.J., Jones, T.D., Baker, M.P. (2010). Prediction of immunogenicity of therapeutic proteins: validity of computational tools. *Biodrugs*, *24* (1), 1–8.

33. Koren, E., De Groot, A.S., Jawa, V., Beck, K.D., Boone, T., Rivera, D., Li, L., Mytych, D., Koscec, M., Weeraratne, D., Swanson, S., Martin, W. (2007). Clinical validation of the "in silico" prediction of immunogenicity of a human recombinant therapeutic protein. *Clin Immunol*, *124*, 26–32.

34. De Groot, A.S., Scott, D.W. (2007). Immunogenicity of protein therapeutics. *Trends Immunol*, *28* (11), 482–490.

35. Brand, C.M., Leadbeater, L., Bellati, G., Marotta, F., Ideo, G. (1993). Antibodies developing against a single recombinant interferon protein may neutralize many other interferon-a subtypes. *J Interferon Res*, *13*, 121–125.

36. Werdelin, O., Meldal, M., Jensen, T. (2002). Processing of glycans on glycoprotein and glycopeptide antigens in antigen presenting cells. *PNAS*, *99* (15), 9611–9613.

37. Green, R.S., Stone, E.L., Tenno, M., Lehtonen, E., Farquhar, M.G., Marth, J.D. (2007). Mammalian *N*-glycan branching protects against innate immune self-recognition and inflammation in autoimmune disease pathogenesis. *Immunity*, *27*, 308–320.

38. Gribben, J.G., Devereux, S., Thomas, N.S., et al. (1990). Development of antibodies to unprotected glycosylation sites on recombinant human GM-CSF. *Lancet*, *335*, 434–437.

39. Dzhambazov, B., Holmdahl, M., Yamada, H., Lu, S., Vestberg, M., Holm, B., Johnell, O., Kihlberg, J., Holmdahl, R. (2005). The major T cell epitope on type II collagen is glycosylated in normal cartilage but modified by arthritis in both rats and humans. *Eur J Immunol*, *35*, 357–366.

40. Rudd, P.M. (2001). Glycosylation and the immune system. *Science*, *291*, 2370–2376.

41. Van Dyken, S.J., Locksley, R.M. (2007). Altered self-*N*-glycans trigger innate-mediated autoimmunity. *Immunol Cell Biol*, *85*, 527–574.

42. Chung, C.H., Mirakhur, B., Chan, E., Le, Q-T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A.E. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-a-1,3-galactose. *N Engl J Med*, *358* (11), 1109–1117.

43. Armstrong, J.K., Hempel, G., Koling, S., Chan, L.S., Fisher, T., Meiselman, J.H., Garratty, G. (2007). Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*, *110*, 103–111.

44. Brennan, F.R., Morton, L.D., Spindeldreher, S., Kiessling, A., Allenspach, R., Hey, A., Muller, P.Y., Frings, W., Sims, J. (2010). Safety and immunotoxicity assessment of immunomodulatory monoclonal antibodies. *MAbs*, *2* (3), 233–255.

45. Niebecker, R., Kloft, C. (2010). Safety of therapeutic monoclonal antibodies. *Curr Drug Saf*, Jul 2 [Epub ahead of print].

46. Descotes, J. (2009). Immunotoxicity of monoclonal antibodies. *MAbs*, *1* (2), 104–111.

47. Herzyk, D.J. (2003). The immunogenicity of therapeutic cytokines. *Cur Op Mol Ther*, *5* (2), 176–171.

48. Wadhwa, M., Skog, A.-L. H., Bird, C., Ragnhammar, P., Lilljefors, M., Gaines-Das, R., Mellstredt, H., Thorpe, R. (1999). Immunogenicity of granulocyte–macrophage colony-stimulating factor (GM-CSF) products in patients undergoing combination therapy with GM-CSF. *Clin Cancer Res*, *5*, 1353–1361.
49. Thirumangalathu, R., Krishnan, S., Ricci, M.S., Brems, D.N., Randolph, T.W., Carpenter, J.F. (2009). Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. *J Pharm Sci*, *98* (9), 3176–3181.
50. Chi, E.Y., Weickmann, J., Carpenter, J.F., Manning, M.C., Randolph, T.W. (2005). Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation. *J Pharm Sci*, *94* (2), 256–274.
51. Tyagi, A.K., Randolph, T.W., Dong, A., Maloney, K.M., Hitscherich, C. Jr, Carpenter ,J.F. (2009). IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles. *J Pharm Sci*, *98* (1), 94–104.
52. Jiang, Y., Nashed-Samuel, Y., Li, C., Liu, W., Pollastrini, J., Mallard, D., Wen, A.Z., Fujimori, K., Pallitto, M., Donahue, L., Chu, G., Torraca, G., Vance, A., Mire-Sluis, T., Freund, E., Davis, J., Narhi, L. (2009). Tungsten-induced protein aggregation: solution behavior. *J Pharm Sci*, *98*, 4695–4710.
53. Richard, J., Prang, N. (2010). The formulation and immunogenicity of therapeutic proteins: product quality as a key factor. *IDrugs*, *13* (8), 550–558.
54. Rathore, N. (2008). Current perspectives on stability of protein drug products during formulation, fill and finish operations. *Biotechnol Prog*, *24*, 504–514.
55. Hochuli, E. (1997). Interferon immunogenicity: technical evaluation of interferon-a2a. *J Interferon Cytokine Res*, *17* (Suppl. 1), S15–S21.
56. Ryff, J.C. (1997). Clinical investigation of the immunogenicity of interferon-a2a. *J Interferon Cytokine Res*, *17* (Suppl. 1), S29–S33.
57. Sharma, B. (2007). Immunogenicity of therapeutic proteins. Part 2. Impact of container closure. *Biotech Adv*, *25*, 318–324.
58. Rosenberg, A.S., Worobec, A.S. (2004). A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 2. Considering host-specific and product-specific factors impacting immunogenicity. *BioPharm Int*, *1*, 34–40.
59. Ryan, M.H., Heavner, G.A., Brigham-Burke, M., McMahon, F., Shanahan, M.F., Gunturi, S.R., Sharma, B., Farrell, F.X. (2006). An in vitro model to assess factors that may stimulate the generation of an immune reaction to erythropoietin. *Int Immunopharmacol*, *6*, 647–655.
60. Schmidt, M., Raghavan, B., Muller, V., Vogl, T., Fejer, G., Tchaptchet, S., Keck, S., Kalis, C., Nielsen, P.J., Galanos, C., Roth, J., Skerra, A., Martin, S.F., Freudenberg, M.A., Goebeler, M. (2010). Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nature Immunol 11*, 814–819 [Epub ahead of print].
61. Kroon, D.J., Baldwin-Ferro, A., Lasan, P. (1992). Identification of sites of degradation in a therapeutic monoclonal antibody by peptide mapping. *Pharm Res*, *9*, 1386–1393.
62. Gregory, R., Edwards, S., Yateman, N.A. (1991). Demonstration of insulin transformation products in insulin vials by high-performance liquid chromatography. *Diabetes Care*, *14*, 42–48.

63. Barbbosa, M.D., Vielmetter, J., Chu, S., Smith, D.D., Jacinto, J. (2006). Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity. *Clin Immunol*, *118*, 42–50.

64. Schernthaner, G., Morkenstein, M., Fink, M., Mayr, W.R., Menzel, J., Schober, E. (1983). Immunogenicity of human insulin (Novo) or pork monocomponent insulin in HLA-DR-typed insulin-dependent diabetic individuals. *Diabetes Care*, *6* (Suppl. 1), 43–48.

65. Fakharzadeh, S.S., Kazazian, H.H. Jr. (2000). Correlation between Factor VIII genotype and inhibitor development in hemophilia A. *Sem Thromb Hem*, *26* (2), 167–171.

66. Kishnani, P.S., Goldenberg, P.C., DeArmey, S.L., Heller, J., Benjamin, D., Young, S., Bali, D., Smith, S.A., Li, J.S., Mandel, H., Koeberl, D., Rosenberg, A., Chen, Y-T. (2010). Cross-reactive immunologic material status affects treatment outcomes in Pompe disease infants. *Mol Genet Metab*, *99*, 26 -33.

67. Junzhi, L., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kutter, D. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.

68. Steis, R.G., Smith, J.W. 2d, Urba, W.J., Clark, J.W., Itri, L.M., Evans, L.M., Schoenberger, C., Longo, D.L., et al. (1988). Resistance to recombinant interferon-alpha-2a in hairy cell leukemia associated with neutralizing anti-interferon-antibodies. *N Engl J Med*, *318*, 1409.

69. Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., et al. (1998). Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum*, *41*, 1552–1563.

70. Baert, F., Noman, M., Vermeire, S., Van Assche, G., D'Haens, G., Carbonez, A., et al. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med*, *348*, 601–608.

71. Pozzilli, C., Antonini ,G., Bagnato, F., et al. (2002). Monthly corticosteroids decrease neutralizing antibodies to IFNb-1b: a randomized trial in multiple sclerosis. *J Neurol*, *249*, 50–56.

72. Antonelli, G., Giannelli, G., Currenti, M., Simeoni, E., Del Vecchio, S., Maggi, F., Pistello, M., Roffi, L., Pastore, G., Chemello, L., Dianzani, F. (1996). Antibodies to interferon (IFN) in hepatitis C patients relapsing while continuing recombinant IFN-α2 therapy. *Clin Exp Immunol*, *104*, 384–387.

73. Giovannoni, G. (2003). Strategies to treat and prevent the development of neutralizing anti-interferon-b antibodies. *Neurology*, *61* (Suppl. 5), S13–17.

74. Bertolotto, A., Malucchi, S., Sala, A., et al. (2002). Differential effects of three interferon betas on neutralizing antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory. *J Neurol Neurosurg Psychiatry*, *73*, 148–153.

75. Carpenter, J.F., Randolph, T.W., Jiskoot, W., Crommelin, D.J., Middaugh, C.R., Winter, G., Fan, Y.X., Kirshner, S., Verthelyi, D., Kozlowski, S., Clouse, K.A., Swann, P.G., Rosenberg, A., Cherney, B. (2009). Overlooking subvisible particles in therapeutic proteins products: gaps that may compromise product quality. *J Pharm Sci*, *98* (4), 1201–1205.

76. Sharma, B. (2008). Immunogenicity of therapeutic proteins. Part 3. Impact of manufacturing changes. *Biotech Adv*, *25*, 325–331.

77. Steis, R.G., Smith, J.W. II, Urba, W.J., Venzon, D.J., Longo, D.L., Barney, R., Evans, L.M., Itri, L.M., Ewel, C.H. (1991). Loss of interferon antibodies during prolonged continuous interferon-a2a therapy in Hairy Cell Leukemia. *Blood*, *77*, 792–798.

78. Kakavanos, R., Turner, C.T., Hopwood, J.J., Kakkis, E.D., Brooks, D.A. (2003). Immune tolerance after long-term enzyme-replacement therapy among patients who have mucopolysaccharidosis I. *Lancet*, *361*, 1608–1613.

79. Pirazzoli, P., Cacciari, E., Mandini, M., Cicognani, A., Zucchini, S., Sganga, T., Capelli, M.. (1995). Follow-up of antibodies to growth hormone in 210 growth hormone–deficient children treated with different commercial preparations. *Acta Paediatr*, *84*, 1233–1236.

80. Underwood, L.E., Voina, S.J., Van Wyk, J.J. (1974). Restoration of growth by growth hormone (Roos) in hypopituitary dwarfs immunized by other hGH preparations: clinical and immunological studies. *J Clin Endocrinol Metab*, *38*, 288–297.

81. Herndon, R.M., Rudick, R.A., Munschauer, F.E. 3rd, Mass, M.K., Salazar, A.M., Coats, M.E., Labutta, R., Richert, J.R., Cohan, S.L., Genain, C., Goodkin, D., Toal, M., Riester, K. (2005). Eight-year immunogenicity and safety of interferon beta-1a–Avonex® treatment in patients with multiple sclerosis. *Mult Scler*, *11*, 409

82. Gneiss, C., Koudouovoh-Tripp, P.M., Popele, S., Gotwald, T., Ehling, R., Lutterotti, A., Aichner, F., Ladurner, G., Eggers, C., Schautzer, F., Kunz, B., Millonig, A., Aspeck, E., Reindl, M., Berger, T., Fazekas, F., Deisenhammer, F. (2009). Influence of interferon-beta therapy switching on neutralizing antibody titers: results from the Austrian Switch Study. *Mult Scler*, *15* (12), 1481–1488.

83. Lallemand, C., Meritet, J.-F., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., Lebon, P., Tovey, M.G. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.

84. Brady, R.O., Murray, G.J., Oliver, K.L., Leitman, S.F., Sneller, M.C., Fleisher, T.A., Barton, N.W., and collaborators. (1997). Management of neutralizing antibody to ceredase in a patient with type 3 Gaucher disease. *Pediatrics*, *100*, E11.

85. Berntorp, E., Shapiro, A., Astermark, J., Blanchette, V.S., Collins, P.W., Dimichele, D., Escuriola, C., Hay, C.R., Hoots, W.K., Leissinger, C.A., Negrier, C., Oldenburg, J., Peerlinck, K., Reding, M.T., Hart, C. (2006). Inhibitor treatment in haemophilias A and B: summary statement for the 2006 international consensus conference. *Haemophilia*, *12* (Suppl. 6), 1–7.

3

GUIDANCE ON IMMUNOGENICITY ASSESSMENT OF BIOLOGICALLY DERIVED THERAPEUTIC PROTEINS: A EUROPEAN PERSPECTIVE

Meenu Wadhwa and Robin Thorpe

3.1. INTRODUCTION

Despite the therapeutic benefits of biological medicines, the unexpected and unpredictable unwanted immunogenicity of such medicines continues to be a major safety concern and a significant challenge for the biotechnology industry and regulatory authorities worldwide. This is because the potential risk of immunogenicity continues throughout the life cycle of a product (e.g., at different phases of clinical development and also postapproval). For example, the issue of immunogenicity arises when the manufacturer of a particular product introduces a change in the product (e.g., expression system, site, manufacturing process, formulation) resulting in a new product for which an assessment of comparability is required [1]. Another important situation in which unwanted immunogenicity needs to be considered is when the patent of an innovator product expires and a "biosimilar product," as defined by the European Medicines Agency (EMA), is developed by a new manufacturer [2–4]. In this case, the new manufacturer develops the new product based on the

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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innovator product (the reference product) but employs its own manufacturing process, purification methods, and testing procedures to develop and evaluate the product in terms of its quality, safety, and efficacy. Even if the noninnovator product has the same gene sequence and is produced using the same vector and host cell line as the marketed innovator protein, the culture conditions, purification methods, and details of the procedures employed will be inevitably different from those used by the innovator, increasing the possibility that the new product generated is different in its biological and clinical profile from the innovator product [2–4]. Consequently, the issue of unwanted immunogenicity has important implications for the success or failure of biotherapeutic products.

While immunogenicity is expected following treatment with nonhuman sequence proteins, it is now well established that many human sequence biologicals also elicit immune responses in some recipients. The issue of unwanted immunogenicity is, however, complex as the incidence of antibody formation varies greatly between proteins and also between products based on the same protein and, in some cases, even with products that have the same amino acid sequence. For example, the incidence of antibodies in multiple sclerosis patients treated with IFN-β1a products varies from 2% to 24% depending on the product used [5]. This is because the immune response to a therapeutic protein is determined by a myriad of factors that in concert induce an immune response which may or may not impact on the clinical outcome. These factors include the structure of the therapeutic (e.g., native sequence or mutein, structural similarity with native protein), its composition (impurities, aggregates, degraded, clipped or deamidated forms), the formulation, dose, frequency and route of administration, duration of treatment, and the biological activities of the protein. In addition, patient-related factors such as the patient's age, genetic predisposition to mounting an immune response, immune status, underlying disease, and use of concomitant medication also can contribute to development of immunogenicity.

Such multifaceted immune responses are associated with variable clinical consequences depending on the product and other factors. In a majority of cases, the immune responses induced are bereft of clinical relevance. However, in some instances, the induced antibodies can have undesirable effect(s), ranging from mild to severe adverse "anaphylactoid" reactions or, in some cases, compromising clinical efficacy by altering biodistribution or by neutralizing the biological function(s) of the therapeutic [6–8]. In rare cases, severe life-threatening immune responses can be induced. Examples include the development of pure red cell aplasia in chronic kidney disease patients treated with an approved erythropoietin product (Eprex), the severe thrombocytopenia caused to normal individuals receiving a pegylated truncated thrombopoietin [megakaryocyte growth and development factor (MGDF)] product (in trials), and clinical problems in hemophilia A patients receiving coagulation factor VIII concentrates [6, 9, 10]. All these problems occurred due to formation of neutralizing antibodies against the biotherapeutic, and patients required

rescue therapies to overcome these immunogenicity-related consequences. It is therefore essential to recognize that the immunogenicity of a biotherapeutic is an important element that needs to be integrated with the quality, nonclinical, and clinical assessments of the product. Studies involving immunogenicity need to be purposely designed to address this issue and further correlated with parameters such as pharmacokinetics, pharmacodynamics, and clinical effects to enable a detailed understanding of the clinical significance of the induced antibodies.

The incidents highlighted here have provoked serious concern among regulators and prompted the Committee for Medicinal Products for Human Use (CHMP) at the EMA to issue a detailed guideline providing background information, general recommendations on immunogenicity from a regulatory and European marketing authorization perspective, and extensive detail on assessment of immunogenicity [11]. This guideline was issued in draft form in January 2007; following consultation and revision within the scientific drafting group, it was adopted by the CHMP in December 2007 and came into operation in April 2008. It is likely that further detailed guidance on certain product classes will be required in future. Consistent with this, an EMA guideline on immunogenicity assessment of monoclonal antibodies (mAbs) intended for *in vivo* clinical use is expected soon, following consultation [12]. More recently, the U.S. Food and Drug Administration (FDA) has also issued draft guidance on immunogenicity [13] taking into account the recommendations of several white papers on the clinical assessment of unwanted immunogenicity published by a consortium of members from industry and elsewhere [14–17].

This chapter provides an overview of the current status, strategies, and challenges related to the immunogenicity assessment of protein therapeutics while focusing on the bioanalytical methodologies required for evaluation of immunogenicity as outlined in the EMA guideline.

3.2. FACTORS CONTRIBUTING TO IMMUNOGENICITY

While immunogenicity may be triggered by any of the multitude of factors that influence an immune response (some are listed in Table 3.1), a consideration of these various risk factors is important for devising strategies to evaluate and minimize the risk of immunogenicity. As such, it is not surprising that protein therapeutics with nonhuman sequences, for example, murine mAbs, that are recognized as foreign antigens by the human immune system elicit an immune response. With improvements in design and introduction of chimeric mouse–human mAbs, followed by humanized and fully human mAbs, there has been a notable decline in immune responses associated with the replacement of murine with human amino acid sequences. However, a mAb with a fully human amino acid sequence can also be immunogenic, as illustrated by the example of adalimumab (Humira, Abbott), a fully human mAb directed

TABLE 3.1 Factors Influencing the Unwanted Immunogenicity of Therapeutic Proteins

Some Factors Determining the Immunogenicity of Therapeutic Proteins
• Product-related
• Product characteristics, molecular structure, variation in amino acid sequence and/or glycosylation (relative to native protein), novel epitopes, aggregates, degradation products, oxidation, deamidation
• Product formulation, container- and closure-derived substances
• Product properties, e.g., immunostimulant/immunosuppressant; replacement therapy, therapy alone/combination therapy; physiologically important, i.e., has endogenous counterpart or redundant
• Process-related
• Process-related impurities, host-cell proteins, other contaminants, process change
• Patient- and disease-related
• Dosing—single/multiple injections, high/low dose, duration
• Route of administration—intravenous/intramuscular/subcutaneous
• Patient's genetic factors, immune status, age, any concomitant medication
• Patient's disease state—acute/chronic, autoimmune/oncology, immunosuppressed
• Previous exposure to therapeutic

against tumor necrosis factor alpha (TNF-α) that has a reported incidence of antibody formation of 12% in adult rheumatoid arthritis patients (Humira package insert, available at www.rxabbott.com/pdf/humira.pdf).

While an understanding of potential risk factors and how to mitigate them is crucial for limiting immunogenicity problems this can, in some instances, be challenging. For example, despite extensive research into the underlying cause of the upsurge in cases of pure red cell aplasia in patients treated with Eprex, identification of the predominant trigger from the numerous potential factors that have been postulated has been difficult. These include change of product formulation and issues relating to failures of cold chain, rubber stopper interaction, the presence of aggregates and leachates in the product, and the route of administration.

The administration route can be important as both subcutaneous and intramuscular administration are often associated with a higher incidence of an immune response in comparison with intravenous administration. The change of the route of administration from subcutaneous to intravenous reduced the incidence of antibody formation in Eprex-treated patients. Multiple (as opposed to single) injections and longer exposure are often associated with an increased risk of an immune response. Re-exposure to the therapeutic following a long interval can trigger a potent immune response. Other patient-related factors such as concomitant therapy and disease are also important in the elicitation of an immune response. For example, 12% of patients with Crohn's disease treated with certolizumab as maintenance therapy developed antibodies, but the incidence of antibody formation decreased to 2% in patients treated with a combination of the antibody and immunosuppressive agents

[18]. In addition, rituximab, which depletes B cells (and should reduce antibody formation), is completely nonimmunogenic in patients with non-Hodgkin's lymphoma but when given to patients with the autoimmune disease systemic lupus erythematosus (SLE) induced antibody formation in 65% of the treated patients [19]. In another example, infliximab, a chimeric anti-TNF mAb product, at a dose of 3 mg/kg bodyweight in combination with methotrexate showed a different immunogenicity profile in adults with rheumatoid arthritis compared with children and adolescents with juvenile idiopathic arthritis who received the same dose. The latter showed a fivefold higher incidence of an immune response than adults, possibly due to differences in protein metabolism. These data, however, should be interpreted with caution as antibody analysis in this study may have been hampered due to interference caused by free infliximab [20]. These examples highlight the need for immunogenicity studies to be conducted if possible not only in the disease targeted by the therapeutic but also in the most sensitive patient population. However, care with comparisons is needed as it is difficult to make valid comparisons unless studies are carried out in the same facility using the same assays, sampling, patient groups, and so on.

3.3. CAN IMMUNOGENICITY BE PREDICTED?

A frequently asked important question is "Can the unwanted immunogenicity of a biotherapeutic product and its immunogenicity profile be determined using a nonclinical animal model?" Clearly, the ability to predict immunogenicity of protein therapeutics prior to their clinical evaluation would be highly advantageous. However, most human proteins (due to their foreign nature) are immunogenic in animals [exceptions are very highly conserved proteins, e.g., transforming growth factor beta (TGF-β)]. This includes nonhuman primates, often claimed to have a higher tolerance to human proteins in comparison with lower mammals. Both granulocyte–macrophage colony-stimulating factor (GM-CSF) and insulin showed a high immunogenicity in animals but a lower immunogenicity in humans. This scenario is often encountered with most biotherapeutics although the reverse may also be apparent. Consequently, animal models are not suitable for predicting the immunogenicity of a biotherapeutic and its clinical consequences in human subjects.

Nevertheless, animal models cannot be completely disregarded, as repeat-toxicity studies conducted in animals provide useful toxicology data that should be interpreted in the context of immunogenicity assessment for any changes in toxicity, efficacy, and pharmacokinetics. In addition, animal models are useful for assessing relative differences in immunogenicity as part of a comparability exercise (i.e., between innovator and biosimilar product, routes of administration, dosing schedules, formulations) and even to evaluate the safety of novel therapeutics for any serious physiological effects (including neutralization of endogenous counterpart) using homologous proteins.

The CHMP guideline encourages the use of emerging technologies (novel *in vivo*, *in vitro*, and *in silico* models) for predicting immunogenicity. Consistent with this, progress has been made toward developing immune-tolerant transgenic mouse models carrying the genes encoding human insulin, IFN-α, and IFN-β [21, 22]. In addition, diverse methods for determination of T-cell epitopes in T-cell assays (using T cells from naive or previously exposed individuals), coupled with *in silico* approaches for sequence analysis and identification of T-cell epitopes, have been developed or are currently in development [19, 23]. These are valuable for identifying immunogenic proteins and selecting those with a minimal risk of immunogenicity. However, "proof of principle" of these methods, which includes producing data on antibody assessment and information on clinical outcome in patients in prospective clinical trials, is urgently needed prior to the application of these tools as useful predictors of a product's immunogenicity and its clinical relevance.

3.4. STRATEGY AND ASSAY PLATFORMS ARE CRITICAL FOR ASSESSMENT OF IMMUNOGENICITY

Although immunogenicity induced by biotherapeutics can comprise both humoral and cellular responses, the most technically feasible approach for assessing immunogenicity currently is the detection of the humoral response, that is, by detecting, measuring, and characterizing antibodies generated against the product. Consequently, testing for antibodies in sera from recipients of a biotherapeutic enrolled in properly planned immunogenicity studies both preapproval and postapproval is the only current realistic option for immunogenicity assessment. A multitiered bioanalytical testing strategy that incorporates a panel of assays is essential for antibody testing and characterization. The strategy often includes an initial evaluation of samples in a screening assay aimed at detecting all antibody-positive samples. As no assay is perfect and false negatives must be avoided, most assays will therefore produce some false positives. A confirmatory step for distinguishing between true and false positive samples is therefore usually needed. In addition, evaluation of positive samples in a neutralizing-antibody assay for assessing the neutralizing capacity of the induced antibodies is also usually required. The strategy can be varied by the applicant as long as all the testing criteria (screening, confirmatory, and neutralization assays) are fulfilled; for example, a manufacturer may decide to test all samples in neutralization assays, rather than selectively testing only those that have been identified as confirmed antibody-positives in the confirmatory assay. Further data on characterization such as antibody class and subclass may be useful or necessary in some cases. In addition, data on pharmacokinetics and/or pharmacodynamics and clinical effects in patients are also required to determine how these correlate with the antibody responses, to gain a detailed understanding of the clinical significance of the induced antibodies.

It is necessary to devise a study strategy prior to embarking on immunogenicity studies for a biotherapeutic. In developing a study strategy, it is critical to consider the risk of generating an immune response, the potential severity of the induced response, and the risk and benefit to the target population. This assessment can have implications on issues relating to timing and frequency of sample collection, sample volumes, assay types and/or formats, and so on, and also may influence decisions relating to assay strategy. Generally, investigators implement their own in-house policies regarding assay strategy based on the risk level and/or development phase of the product. Some implement neutralization assays at the clinical phase after considering the risk of immunogenicity while others conduct neutralization assays at both preclinical and clinical level irrespective of the risk associated with the product. A strategy using a risk-based approach for the product in different phases of development, preclinical and clinical, is pertinent and justified [24].

A frequently asked question is "Are assays for assessing cell-mediated immune responses required for assessment of immunogenicity." At present, the view taken by most regulators is that these assays are normally not required for marketing authorization, as they are still emerging as valuable tools for characterizing and evaluating immunogenicity. The development and need for such assays is, therefore, normally considered case by case. Examples of assays that can be used for detection and assessment of cell-mediated immune responses include T-cell stimulation–proliferation assays and cytokine production–release assays using enzyme-linked immunosorbent assays (ELISAs), enzyme-linked immunospot (ELISPOT), and flow cytometric procedures.

3.5. ANTIBODY DETECTION ASSAYS

3.5.1. Screening Assays

With new platforms continually being exploited to enhance the ability to detect therapeutic-induced antibodies in sera of treated patients, there are various possibilities that can be used for screening antibodies. These include conventional approaches such as ELISAs, radioimmunoprecipitation assays, and other technologies—which include surface plasmon resonance (SPR), time-resolved fluorescence resonance energy transfer (FRET), electrochemiluminescence (ECL), and dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) [25–28]. However, each of these methods has benefits and limitations that need to be considered prior to choosing them for assay purposes [29]. In our experience, the format of the screening assay is very much dependent on the therapeutic under study. For example, for determining antibodies against a nonimmunoglobulin recombinant protein, all of the available platforms are suitable options but need to be judged on their relative merits. However, in the case of a humanized or fully human immunoglobulin

therapeutic, the choices for detection of human anti-human antibodies are fairly limited and the suitability of a particular platform and/or assay format is very much dependent on the antibody itself [30, 31].

Since all antibodies need to be detected by the screening assays used, in some instances, should the initial assay selection not meet desired expectations (e.g., give poor sensitivity or high backgrounds), other procedures may need to be developed and possibly evaluated prior to selecting an assay for use in clinical trials. For example, for the large number of clinical samples anticipated in many studies, platforms that allow high throughput are highly preferable in comparison with those where throughput is limited. It is worthwhile noting that not all assay formats enable detection of different classes and subclasses of antibodies. Detection of transient or low-affinity antibodies with rapid off-rates that may be clinically significant is also important; such antibodies are easily missed by techniques such as ELISAs, due to antibody losses during the washing steps [32]. In some cases, therefore, assays may need to be adapted to allow detection of these antibody types, or alternative real-time methods such as SPR that allow detection of antibodies with rapid off-rates should be used [32].

Although screening assays should have the ability to identify all positive samples irrespective of their dissociation rates and isotype, they should not provide false negative results nor underestimate true positives. Initially, some samples may be classified as positive even though they do not contain antigen-specific antibody, that is, are false positives. It is important to realize that a screening assay which is unable to identify any positives following a preclinical or clinical trial casts doubt on the ability of the assay to detect low positive samples.

3.5.2. Confirmatory Assays

As stated earlier, a confirmatory step is required for eliminating false positive samples. Any of the methods that are employed as a screening assay can be used for this purpose with the inclusion of an immunodepletion step. For immunodepletion, the serum sample (identified as positive by the screening assay) is assayed in the absence or presence of excess antigen such that the latter sample no longer provides a positive signal for "real" positive samples in the screening assay. Care should be taken in the interpretation of such results, as some substances may also cross-react and bind nonspecifically. Although other assay formats can also be used that are based on a different scientific principle and take into account the limitations and characteristics of the screening assay, it is not normally necessary to select a different assay; the screening assay with an additional confirmatory step (e.g., immunodepletion) is normally adequate.

Antibodies present in confirmed positive samples may, in some instances, have specificity for other product- or process-related components (e.g., contaminants), which can cause data to be misinterpreted. For example, very low

levels of expression system derived bacterial proteins in rDNA products can cause significant antibody development whereas the major protein present (the active principle) can be less immunogenic [33]. Testing for cross-reactivity with other products based on the same protein as well as its endogenous counterpart (if possible and relevant) is useful for discriminating between specific and nonspecific antibodies [34].

If antibodies are confirmed, these need to be characterized in order to establish their clinical significance. This involves assessment of their characteristics in terms of the antibody content, specificity, antibody class and subclass, affinity, and, most important, evaluation of their ability to neutralize the biological effects of the therapeutic in an *in vitro* assay.

3.5.3. Neutralizing Antibody Assays

Testing antibodies in a neutralizing antibody assay is an important element of immunogenicity assessment, as results from neutralization assays can be correlated with the effect of antibodies on reducing the clinical response to the product. Specific examples of this include IFN-β, GM-CSF, and Tysabri [5, 33, 34, 35]. In neutralizing antibody assays, the sample (at various dilutions) is preincubated with the therapeutic; if neutralizing antibodies exist in the sample, the therapeutic is unable to bind to its target, resulting in an inhibition of assay response. Use of this approach allows a neutralizing dose response to be determined and the neutralizing capacity of each sample to be calculated [15].

Two types of neutralization assay can be used: a cell-based bioassay and a non-cell-based competitive ligand-binding assay. Bioassays used for defining the potency of different therapeutics can be optimally refined and used as the basis of a neutralization assay. Such assays may use early (e.g., assessment of phosphorylation of intracellular substrates) or late (e.g., induction or secretion of cellular proteins, cell proliferation, or cell death) endpoints [36–41]. The assay formats and designs of cell-based neutralization assays often vary in complexity depending on the type of therapeutic—for example, an agonist (e.g., cytokines, growth factors) or antagonist (e.g., soluble receptors, antagonistic mAbs)—and the type of assay required. Such assays are susceptible to nonspecific matrix effects, and inclusion of a specificity step should be considered where possible. Although commonly used, these assays can be difficult to establish as opposed to non-cell-based competitive ligand-binding assays, which are rapidly gaining momentum as a platform to use at least for some products [42]. The use of cell-based bioassays, however, is generally recommended by regulators as they are considered to be more reflective of the *in vivo* situation, in contrast to non-cell-based competitive ligand-binding assays. If the latter are used, it is important to demonstrate that they appropriately reflect neutralizing capacity.

It is important to develop the assay strategy and ensure that the assay platforms used and parameters selected are satisfactory for the particular product. The applicant has the choice of assay strategy and methods to be used

for the assessment; such decisions should be made early during development to allow adequate time for assay qualification and validation. All assays should be suitable for their intended purpose. Therefore, assay validation and inclusion of relevant antibody/reference controls need to be considered and implemented appropriately. If actions on such measures are taken early, data from early clinical trials may be used to support data from later clinical trials.

3.6. INTERPRETATION OF DATA

It is essential to establish clear criteria for deciding how samples will be considered positive or negative, how positive results will be confirmed, and how levels of antibody will be quantified. Approaches for these can differ depending on the assay design and need to be decided accordingly [43]. Adequate control samples need to be included in all assays to ensure confidence in the results obtained.

Simple classification of samples into two groups (e.g., positive or negative) results in loss of quantitative information that may be useful in some cases and so this approach is not recommended. A variety of statistical approaches are available for classification of data. For example, one may consider receiver operating characteristic (ROC) curve analysis, in which test results depend on the threshold or the cutoff point used to classify the data [44]. It is important to realize that classification groups all results into one of four classes: true positive; false positive; false negative; true negative. In the ROC method, sensitivity and specificity depend on the value of the cutoff point, and a plot of sensitivity against specificity provides the true positive rate against a false positive rate. An ideal ROC provides 100% sensitivity (all true positives found) and 100% specificity (no false positives) but this is impossible to achieve in practice.

Quantification, in comparison with classification, is more complex to analyze and interpret. However, it is very useful as it allows ordering and clustering of data and also correlation of results. Quantification in terms of a "similar" antibody preparation (an "antibody standard," also sometimes "antibody reference standard/preparation") is difficult to achieve, as the reference antibody standards are unlikely to reflect the antibody affinities, avidities, proportions, and other characteristics of test sera. This makes it difficult, if not impossible, to have direct valid comparisons between samples and reference antibody, especially on a mass basis. This implies that calibration of such assays should be carried out using an acceptable, valid approach that is clearly described. Often the best option is to report data as a titer based on a standard, defined procedure for calculating this value.

In some instances, it may be necessary to use an antibody standard for expressing relative levels of antibodies in the test samples. Antibody levels may be expressed using arbitrary units providing that the unitage has been well defined for the reference material. Although not ideal, the use of a

common antibody standard is a pragmatic approach that may provide comparable estimates for antibody levels in the samples "relative" to the standard and reduce variability. Significant correlations (consistent ranking of samples) provide confirmation that different assays (and different laboratories and methods) measure the same factor(s), indicating that use of a standard may enhance comparisons (between assays, laboratories, methods). Therefore this strategy may, in some instances, be crucial if some problems, notably noncomparability between assays, are to be avoided. This situation is most likely to occur when a number of sequential samples from the same animal or patient are available and it is difficult to include all samples from all patients in the same assay runs for establishing a valid comparison of antibody levels between different samples or patients.

3.6.1. Assessment of Binding Antibodies

For assessment of binding antibodies, the cutoff-point method is commonly used. The assay cutoff point is the level of response of the assay or the assay threshold at or above which a sample is considered positive and below which it is considered negative [14]. The cutoff point is therefore determined by establishing the nonspecific background of the assay by testing a number of appropriate negative control samples and then deciding statistically (e.g., usually three standard deviations above background value) or on the basis of real data (e.g., at least twice the mean value obtained for the background) what will be considered the lowest positive result.

When determining a cutoff point, it is important to use test samples as similar as possible to the patient samples from the target disease; sera from normal healthy individuals is not a valid option. Therefore, an assay baseline or a statistically valid background value would need to be established using pretreatment samples from patients (or from some other valid, relevant diseased population) for categorizing samples as either antibody-positive or antibody-negative. For example, samples from both "healthy controls" and study subjects with the disease need to be assessed if the assay will be used for both of these populations. It should be noted that it may be necessary to define different cutoff points depending on the disease or patient population being tested. As assay signal responses tend to vary, the cut-point value may also need to be altered and so a procedure to account for this needs to be adopted. This has been explained in detail [14].

While the cutoff point approach is very useful and has been extensively adopted for antibody assays by the biotech industry, it has several problems. The main problem is that with a "good" assay (particularly a bridging assay), there is a low matrix interference that results in a very low cutoff point. Many investigators use the negative control to normalize the cutoff point for interassay variability. It has been suggested to use the 99% confidence interval as an upper acceptance limit for the negative control to assure that an unusually high response of the negative control does not compromise the sensitivity of

the screening assay. However, for low cutoff points, it is almost impossible to apply this acceptance criterion. Moreover, these very low cutoff points can provide a significantly high false positive rate (>5%) in a study as there is virtually no difference between absorbance values of 0.01 and 0.03 in ELISAs.

3.6.2. Assessment of Neutralizing Capacity

After confirming that a sample is positive for binding antibodies, usually the next step is to determine the neutralization potential of the antibodies and the levels of these antibodies in the samples. A pertinent question often asked is "How are the amounts of neutralizing antibodies expressed?" In most published studies, the antibody content is often expressed as a "titer." However, the titer value, which is dependent on a variety of factors, is not consistently defined and can often vary between assays and particularly between laboratories. A recommended approach for expressing the neutralizing activity of a serum sample (containing antibody) is to report results as the amount of serum required to neutralize the biological activity induced by a constant amount of the antigen (34). For example, for GM-CSF, the volume of serum required to neutralize the activity of 10 IU of cytokine can be calculated using serum ED_{50} responses obtained by fitting common asymptotes and slope for all sera analyzed. This approach can also be used to analyze responses to different GM-CSF preparations or products and can be applied to other biologicals. Alternatively, levels of neutralizing antibodies of the samples can also be determined by using an antibody standard for expressing relative levels of antibodies in the test samples. However, this approach is less than ideal and may be flawed for the same reasons previously discussed for binding antibodies.

3.7. VALIDATION OF ASSAYS

Considerable emphasis on the validation of antibody assays is required as these are challenging in various respects (considered later in this section). Assay validation for immunogenicity assessment, however, is an ongoing process. It is not necessary to have a fully validated assay for phase I clinical trials, but the assay should be qualified as fit for the intended purpose and capable of providing reliable and robust data that can be appropriately interpreted and analyzed. It is worthwhile noting that a qualified and validated assay in early development helps to accumulate valuable data on immunogenicity for the application. In any case, a full validation should be achieved prior to initiation of phase III (or pivotal) trials.

It should be realized that the parameters requiring validation are unique to each method and its intended use and therefore must be carefully determined on a case-by-case basis. It is important to conduct appropriate validation studies to show that the assays demonstrate appropriately linear

concentration-dependent responses to relevant analytes and fulfill typical validation characteristics, which include accuracy, precision, repeatability, specificity, and sensitivity. Assessment of interference if any from the biological itself and other substances present in the sample (e.g. hemoglobin, lipids, soluble receptors) or concomitant medication that might prevent antibodies from being detected should be undertaken where required, and ways to minimize these problems should be considered. Spiking and recovery experiments may assist in providing useful validation information. In certain instances, residual therapeutic present in the sample can bind to the induced antibodies, forming immune complexes that adversely affect the results such that they may be classified as inconclusive or false negative for the presence of antibodies. This is clearly not satisfactory and procedures such as acid dissociation or other procedures, or even a different sampling strategy (e.g., evaluation of samples taken after cessation of treatment and after a washout period), may need to be implemented to facilitate detection of antibodies [45–47].

Development of appropriate reference materials, that is, the use of relevant biological standards and/or well-characterized positive and negative controls is necessary for assay standardization, validation, and calibration. An ideal positive control is a human preparation that has a significant antibody content and is available in sufficient amounts for continued use. Obtaining a human antibody-positive sample during initial product and assay development is challenging. In some cases, pooling of human samples may be considered, although this is not always possible. Therefore, affinity purified antisera from hyperimmunized animals may be the only available option despite their limitations when used in certain types of assays and the different characteristics of these antibodies compared with human antibodies. Such antibodies need to be well characterized and tested in the selected assay matrix. Preparing a panel of preparations containing antibodies with different characteristics (e.g., high and low affinity) and in different amounts (high, middle, and low antibody content) is useful for assessing assay performance and for characterizing and validating assays (Fig. 3.1). For neutralization assays, the preparation should have significant neutralizing activity, but inclusion of a non-neutralizing antibody preparation in the assays is also useful. Negative standards or controls are required for establishing baselines of the assays. While it is common practice to determine the assay baseline by using sera from a panel of healthy individuals, these sera are not representative of the patient population and cannot be substituted as baseline samples for the patients targeted for treatment with the therapeutic. For the clinical cohort, samples need to be taken prior to treatment with the therapeutic to establish the relevant assay baseline; the samples also need to be assessed for pre-existing antibodies or other interfering substances and then compared with the post-treatment samples for data to be interpreted correctly. In some instances, patients may have pre-existing antibodies that react with the product and may cause clinical problems and/or affect immunogenicity assessment. For example, in a recent study, pre-existing IgE antibodies specific for gal-α-1,3-gal, which exist in some human individuals, caused

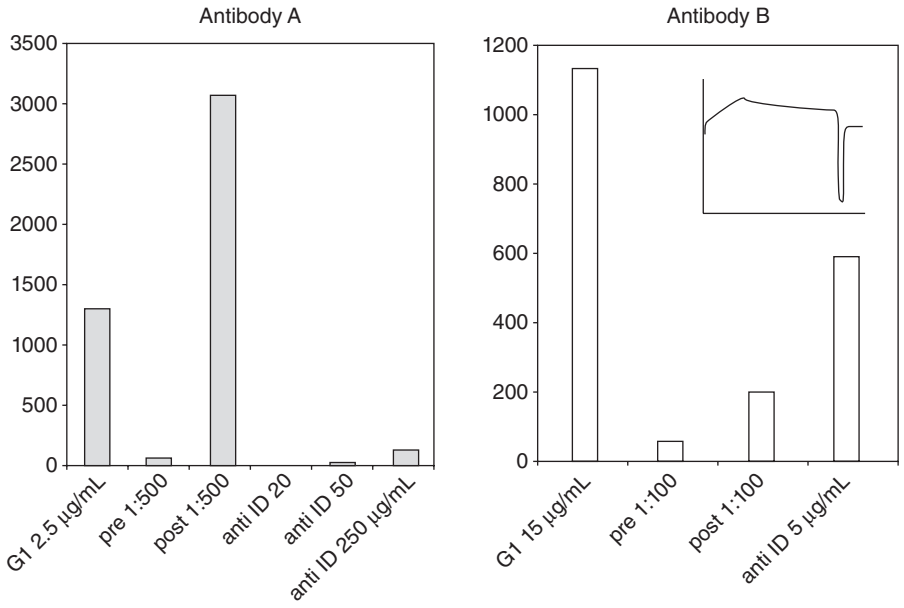

Fig. 3.1 Data (in response units) from a surface plasmon resonance experiment in which two different human mAbs, Antibody A and Antibody B, were immobilized on sensor chips and the binding of a selection of different antibodies was assessed (for these see below). Inset shows the sensorgram obtained with the anti-ID antibody. G1, an IgG1 murine mAb that binds all subclasses of human IgG; pre, animal serum taken prior to immunization with the specific therapeutic antibody; post, animal serum taken after hyperimmunization with the therapeutic antibody; anti ID, idiotypic antibodies specific for the therapeutic antibody.) As expected, G1 binds well to both the antibodies, and the pre serum shows only minimal binding. With specific hyperimmunized animal sera, strong binding is evident with Antibody A and moderate binding with Antibody B. With the idiotypic antibody, however, extremely weak binding is seen with Antibody A even at high concentrations, showing that this assay is incapable of detecting specific antibodies and therefore is unsuitable for use with clinical samples. In the case of Antibody B, the idiotypic antibody provides a very strong response, indicating that the assay is capable of detecting specific antibodies directed against the complementarity-determining region and therefore is suitable for clinical use. These data clearly show that the selection of well-characterized positive control antibodies is important for validation of assays.

hypersensitivity reactions in cetuximab-treated patients as they reacted with the carbohydrate present on the Fab portion of the cetuximab (anti-EGF receptor) molecule [48].

In addition to setting specifications for all assay reagents and materials, it is important to define a strategy for collecting test samples, for example, the conditions for sampling, processing and storage of the samples. Sampling schedules should be adapted and selected individually for each product, taking into account various factors including dose, schedule, and pharmacokinetic

data. A defined sampling schedule in which samples are collected sequentially at predefined time-points prior to product administration during treatment and at baseline is required. Repetitive sampling should be designed to clearly distinguish between transient and persistent antibody responses, as the latter are likely to have a more serious clinical impact. In some instances, additional sampling may be needed, for example, after discontinuation of treatment and/or after a wash-out period. A standardized approach for sampling and testing is valuable for obtaining comparative data from different trials in different indications and should be implemented where possible.

3.8. CONCLUSIONS

The evaluation of unwanted immunogenicity is recognized as an important element of product development. With evolution of new technologies and subsequent development of novel therapeutics and treatment modalities, the assessment of immunogenicity will continue to be a requirement for product approval from a safety as well as an efficacy perspective. Despite continuous enhancements in our understanding of immunogenicity, the issues involved with unwanted immunogenicity still present various challenges and difficulties. Prospective planning and optimization of protocols for immunogenicity studies need to be considered at the outset to avoid inadequacies in data at submission. To assist with this process, the EMA has issued general guidance, which advocates a multidisciplinary risk- and science-based approach for assessment of immunogenicity. While a risk-based strategy is recommended, the guideline does not provide any information on whether or how this should be applied to immunogenicity assessment. The risk of immunogenicity associated with a particular product can alter depending on the situation, as the immune response is multifactorial; this precludes judgments and makes it difficult to define an evaluation strategy based on a risk-dependent approach. The regulatory framework defined within the guidance is flexible as opposed to prescriptive and allows the applicant to use different approaches and strategies for immunogenicity assessment and to modify (as and when necessary) on a case-by-case basis depending on the therapeutic. The use of novel approaches where appropriate in this rapidly evolving area is also actively encouraged. Although flexible, the guidance emphasizes that a systematic and comprehensive evaluation of immunogenicity be undertaken for each biotherapeutic (whether biosimilar or novel) such that adequate, useful data are generated by the applicant. From a regulatory standpoint, data from immunogenicity studies should cover all important aspects of unwanted immunogenicity and consider critical issues, including sampling schedules and strategies employed, impact on pharmacokinetic and pharmacodynamic parameters, and clinical effects in terms of both efficacy and safety. For the latter, the EMA guideline explicitly states that the impact of a product's immunogenicity on patient safety in terms of acute (e.g., infusion or anaphylactic reactions) and unexpected clinical symptoms and nonacute consequences (e.g., delayed

hypersensitivity, immune complex–related effects, and cross-reactivity with an endogenous antigen) should be considered. Where safety issues are suspected, risk mitigation and management plans are needed. Specific recommendations are also clearly stated; for example, immunogenicity should be evaluated in all study participants and not only in symptomatic individuals, and clear criteria need to be defined to distinguish positive from negative findings and to confirm positive results. In addition, since it is recognized that preapproval clinical studies are unlikely to address the low frequency, clinically serious immunogenicity that can occur with biologicals (e.g., erythropoietin), immune monitoring during postapproval clinical studies, as part of pharmacovigilance surveillance, may also be needed to establish the true risk associated with an unwanted immune response. Undoubtedly, implementation of this approach for assessment of unwanted immunogenicity will enable a detailed understanding of the immunogenicity of a product and its clinical significance, and the approach also will be useful for facilitating approval of the wide range of novel biotherapeutics currently in development.

ACKNOWLEDGMENTS

We are thankful to Isabelle Cludts, Biotherapeutics Group, National Institute for Biological Standards and Control (UK), for providing us with the figure on validation of SPR assay.

REFERENCES

1. European Medicines Agency. (2007). Guideline on comparability of biotechnology-derived medicinal products after a change in the manufacturing process. Non-clinical and clinical issues. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/10169506enfin.pdf
2. European Medicines Agency. (2005). Guideline on similar biological medicinal products. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/043704en.pdf
3. European Medicines Agency. (2006). Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/4934805en.pdf
4. European Medicines Agency. (2006). Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/4283205en.pdf
5. Bertolotto, A. (2004). Neutralizing antibodies to interferon beta: implications for the management of multiple sclerosis. *Curr Opin Neurol*, *17*, 241–246.

6. Waters, B., Lillicrap, D. (2009). The molecular mechanisms of immunomodulation and tolerance induction to factor VIII. *J Thromb Haemost*, *7* (9), 1446–1456.
7. Wang, J., Lozier, J., Johnson, G., Kirshner, S., Verthelyi, D., Pariser, A., Shores, E., Rosenberg, A. (2008). Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment. *Nature Biotechnol*, *26* (8), 901–908.
8. Baert, F., Noman, M., Vermiere, S., Van Assche, G., D'Haens, G., Carbonez, A., Rutgeerts, P. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med*, *348*, 601–608.
9. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
10. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red cell aplasia and anti-erythropoietin antibodies against human erythropoietin in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.
11. European Medicines Agency. (2007). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/1432706en.pdf
12. European Medicines Agency. (2009). Concept paper on immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use has been released for consultation. Available at http://www.EMEA.europa.eu/pdfs/human/biosimilar/11472009en.pdf
13. Food and Drug Administration (FDA). (2009). Guidance for industry. Assay development for immunogenicity testing of therapeutic proteins. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf
14. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
15. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
16. Shankar, G., Devanarayan, V., Amaravadi, L., et al. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48* (5), 1267–1281.
17. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333* (1–2), 1–9.
18. Schreiber, S., Khaliq-Kareemi, M., Lawrance, I.C., Thomsen, O.Ø., Hanauer, S.B., McColm, J., Bloomfield, R., Sandborn, W.J. (2007). Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med*, *357*, 239–250.

19. Van Walle, I., Gansemans, Y., Parren, P.W., Stas, P., Lasters, I. (2007). Immunogenicity screening in protein drug development. *Expert Opin Biol Ther*, *7*, 405–418.
20. European Medicines Agency. (2008). European public assessment report (EPAR) for Remicade®. Available at http://www.EMEA.europa.eu/humandocs/Humans/EPAR/remicade/remicade.htm
21. Hermeling, S., Aranha, L., Damen, J., Slijper, M., Schellekens, H., Crommelin, D., Jiskoot, W. (2005). Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b. *Pharm Res*, *22*, 1997–2006.
22. van Beers, M.M., Sauerborn, M., Gilli, F., Hermeling, S., Brinks, V., Schellekens, H., Jiskoot, W. (2010). Hybrid transgenic immune tolerant mouse model for assessing the breaking of B cell tolerance by human interferon beta. *J Immunol Methods*, *352* (1–2), 32–37.
23. De Groot, A.S., Moise, L., McMurry, J.A., Wambre, E., Van Overtvelt, L., Moingeon, P., Scott, D.W., Martin, W. (2008). Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." *Blood*, *112* (8), 3303–3311.
24. Shankar, G., Pendley, C., Stein, K.E. (2007). A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. *Nature Biotechnol*, *25*, 555–561.
25. Gross, J., Moller, R., Henke, W., Hoesel, W. (2006). Detection of anti-EPO antibodies in human sera by a bridging ELISA is much more sensitive when coating biotinylated rhEPO to streptavidin rather than using direct coating of rhEPO. *J Immunol Methods*, *313*, 176–182.
26. Tacey, R., Greway, A., Smiell, J., Power, D., Kromminga, A., Daha, M., Casadevall, N., Kelley, M. (2003). The detection of anti-erythropoietin antibodies in human serum and plasma. Part I. Validation of the protocol for a radioimmunoprecipitation assay. *J Immunol Methods*, *283*, 317–329.
27. Thorpe, R., Swanson, S.J. (2005). Current methods for detecting antibodies against erythropoietin and other recombinant proteins. *Clin Diagn Lab Immunol*, *12*, 28–39.
28. Moxness, M., Tatarewicz, S., Weeraratne, D., Murakami, N., Wullner, D., Mytych, D., Jawa, V., Koren, E., Swanson, S.J. (2005). Immunogenicity testing by electrochemiluminescent detection for antibodies directed against therapeutic human monoclonal antibodies. *Clin Chem*, *51*, 1983–1985.
29. Wadhwa, M., Thorpe, R. (2006). Strategies and assays for the assessment of unwanted immunogenicity. *J Immunotoxicol*, *3*, 115–121.
30. Aarden, L., Ruuls, S.R., Wolbink, G. (2008). Immunogenicity of anti-tumor necrosis factor antibodies—toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20* (4), 431–435.
31. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Stapel, S., Lems, W.F., Aarden, L., Dijkmans, B.A.C., Tak, P.P., Wolbink, G.J. (2007). Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis*, *66*, 921–926.
32. Lofgren, J.A., Dhandapani, S., Pennucci, J.J., Abbott. C.M., Mytych, D.T., Kaliyaperumal, A., Swanson, S.J., Mullenix, M.C. (2007). Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *J Immunol*, *178*, 7467–7772.

33. Wadhwa, M., Skog, A.-L.H., Bird, C., Ragnhammar, P., Lilljefors, M., Gaines-Das, R., Mellstedt, H., Thorpe, R. (1999). Immunogenicity of granulocyte–macrophage colony stimulating factor (GM-CSF) products in patients undergoing combination therapy with GM-CSF. *Clin Can Res*, *5*, 1353–1361.

34. Wadhwa, M., Bird, C., Fagerberg, J., Gaines-Das, R., Ragnhammar, P., Mellstedt, H., Thorpe, R. (1996). Production of neutralizing GM-CSF antibodies in carcinoma patients following GM-CSF combination therapy. *Clin Exp Immunol*, *104*, 351–358.

35. Subramanyam, M. (2008). Case study: immunogenicity of natalizumab. In *Biotechnology: Pharmaceutical Aspects*, Vol VIII. *Immunogenicity of Biopharmaceuticals*. van der Weert M, Moller EH, eds. Springer, New York; AAPS Press.

36. Wei, X., Swanson, S.J., Gupta, S. (2004). Development and validation of a cell-based bioassay for the detection of neutralizing antibodies against recombinant human erythropoietin in clinical studies. *J Immunol Methods*, *293*, 115–126.

37. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., Marnetto, F., Gilli, D. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321* (1–2), 19–31.

38. Hu, J., Gupta, S., Swanson, S.J., Zhuang, Y. (2009). A bioactive drug quantitation based approach for the detection of anti-drug neutralizing antibodies in human serum. *J Immunol Methods*, *345* (1–2), 70–79.

39. Kelley, M., Cooper, C., Matticoli, A., Greway, A. (2005). The detection of anti-erythropoietin antibodies in human serum and plasma. Part II. Validation of a semi-quantitative 3H-thymidine uptake assay for neutralizing antibodies. *J Immunol Methods*, *300*, 179–191.

40. Moore, M., Meager, A., Wadhwa, M., Burns, C. (2009). Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type I interferon–inducible 6–16 mRNA. *J Pharm Biomed Anal*, *49*, 534–539.

41. Lallemand, C.J., Meritet, F., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., et al. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.

42. Baltrukonis, D., Finco-Kent, D., Poirier, M., LeSauteur, L., Kawabata, K. (2006). *Development* and validation of a quasi-quantitative bioassay for neutralizing antibodies against CP-870893. *J Immunotoxicol*, *3*, 157–164.

43. Klakamp, S.L., Lu, H., Tabrizi, M., Funelas, C., Roskos, L.K., Coleman, D. (2007). Application of analytical detection concepts to immunogenicity testing. *Anal Chem*, *79*, 8176–8184.

44. Zwieg, M.H., Campbell, G. (1993). Receiver–operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem*, *39*, 561–577.

45. Patton, A., Mullenix, M.C., Swanson, S.J., Koren, E. (2005). An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J Immunol Methods*, *304*, 189–195.

46. Bourdage, J.S., Cook, C.A., Farrington, D.L., Chain, J.S., Konrad, R.J. (2007). An affinity capture elution (ACE) assay for detection of anti-drug antibody to

monoclonal antibody therapeutics in the presence of high levels of drug. *J Immunol Methods*, *327*, 10–17.

47. Sickert, D., Kroeger, K., Zickler, C., Chokote, E., Winkler, B., Grenet, J.M., Legay, F., Zaar, A. (2008). Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore. *J Immunol Methods*, *334* (1–2), 29–36.
48. Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *N Engl J Med*, *358* (11), 1109–1117.

4

JAPANESE REGULATORY PERSPECTIVE ON IMMUNOGENICITY

Takao Hayakawa and Akiko Ishii-Watabe

Immunogenicity is a critical safety issue in the manufacture and clinical use of protein products and requires special attention. Several major points need to be considered when dealing with immunogenicity concerns; however, it is crucial that known product- and process-related immunogenicity factors, if any, be minimized or eliminated.The immunogenicity of a therapeutic protein product in humans can ultimately only be assessed in clinical studies. In such clinical assessment, it is understood that an observational study of antibody formation against an active ingredient, including the desired product and product-related substances, as well as product-related impurities is very important. Moreover, the potential formation of antibodies against substances derived from the manufacturing process that may be introduced into the final product (i.e., process-related impurities) should be adequately taken into account and carefully monitored. This chapter describes some aspects of immunogenicity concerns about therapeutic protein products in general, introduces some experiences in Japan, and discusses the practical approach to minimizing potential risks in clinical use.

4.1. INTRODUCTION

From 1985 to 2011, more than 70 biotechnological protein products were approved as therapeutics in Japan (Table 4.1). Based on the data from clinical

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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TABLE 4.1 Biotechnology-Derived Protein Products Approved in Japan

Classification	Description	Japanese Accepted Name
Enzyme	t-PA	Alteplase, Pamiteplase, Monteplase
	β-Glucocerebrosidase	Imiglucerase
	α-Galactosidase	Agalsidase Alfa, Agalsidase Beta
	α-L-Iduronidase	Laronidase
	Acid α-glucosidase	Alglucosidase Alfa
	Iduronate-2-sulfatase	Idursulfase
	N-Acetylgalactosamine 4-sulfatase	Galsulfase
	Urate oxidase	Rasburicase
Plasma protein	Factor VIII	Octocog Alfa, Rurioctocog Alfa
	Factor VII (activated)	Eptacog Alfa (Activated)
	Factor IX	Nonacog Alfa
	Thrombomodulin	Thrombomodulin Alfa
	Albumin	Human Serum Albumin
Hormone	Insulin	Insulin Human, Insulin Lispro, Insulin Aspart, Insulin Glargine, Insulin Detemir, Insulin Glulisine
	Growth hormone	Somatropin
	Pegylated growth hormone	Pegvisomant
	Somatomedin C	Mecasermin
	Atrial natriuretic peptide	Carperitide
	Glucagon	Glucagon
	GLP-1 analogue	Liraglutide
	PTH analogue	Teriparatide
	Follicle stimulating hormone	Follitropin Alfa, Follitropin Beta
Cytokine	G-CSF	Filgrastim, Lenograstim, Nartograstim
	IL-2	Celmoleukin, Teceleukin
	bFGF	Trafermin
	Erythropoietin	Epoetin Alfa, Epoetin Beta, Epoetin Kappa
	Erythropoietin analogue	Darbepoetin Alfa
	Interferon alpha	Interferon Alfa (NAMALWA[a]), Interferon Alfa (BALL-1[a]), Interferon Alfa-2b, Interferon Alfacon-1
	Interferon beta	Interferon beta[a], Interferon Beta-1a, Interferon Beta-1b
	Interferon gamma	Interferon Gamma-1a
	Pegylated interferon alpha	Peginterferon Alfa-2a, Peginterferon Alfa-2b
Monoclonal antibody	Anti-CD3 antibody	Muromonab-CD3[a]

TABLE 4.1 (*Continued*)

Classification	Description	Japanese Accepted Name
	Anti-CD20 antibody conjugated with tiuxetan	Ibritumomab Tiuxetan
	Anti-CD20 antibody	Rituximab
	Anti-TNF-α antibody	Infliximab
	Anti-CD25 antibody	Basiliximab
	Anti-EGFR antibody	Cetuximab
	Anti-HER2 antibody	Trastuzumab
	Anti-RS virus antibody	Palivizumab
	Anti-IL6R antibody	Tocilizumab
	Anti-CD33 antibody conjugated with calicheamicin	Gemtuzumab Ozogamicin
	Anti-IgE antibody	Omalizumab
	Anti-VEGF antibody	Bevacizumab
	Anti-VEGF antibody fragment	Ranibizumab
	Anti-TNF-α antibody	Adalimumab
	Anti-C5 antibody	Eculizumab
	Anti-EGFR antibody	Panitumumab
	Anti-IL12/IL23 p40 antibody	Ustekinumab
Fusion protein	TNFR-Fc	Etanercept
	CTLA4-Fc	Abatacept
	Fc-TPOR agonist peptide	Romiplostim

[a]Cell-derived nonrecombinant protein.

applications of protein products in Japan and other countries, it is known that all protein products are immunogenic, even if the products have full human sequences [1–3]. The typical examples in which even human proteins exhibit immunogenicity include recombinant human insulins and interferons (IFNs) [4]. Interestingly, it is known of human insulin that the deviation of one to three amino acid residues or variants generated during manufacture or storage seriously affect their immunogenicity [5, 6]. Both recombinant IFN-α2b and IFN-α2a are subtypes of human IFN that differ at only one amino acid residue, and yet exhibit great differences in immunogenicity [7]. Factors other than protein primary structure can also cause differences in immunogenicity. For example, the immunogenicity of a glycoprotein such as IFN-β can be enhanced by the loss of sugar chains [8–10]. Although the mechanisms by which antibodies are formed against therapeutic human protein products are not fully elucidated, risk factors related to the immunogenicity of these products have been revealed. Both product- and patient-related factors are involved in antibody formation against product components [11].

Since therapeutic protein products often contain detectable amounts of process-related impurities, immunogenicity and adjuvant activity of process-related impurities may need to be considered, in addition to the concerns about antibody formation against active ingredients (including the desired product and product-related substances). For example, host-cell proteins and lipopolysaccharides derived from cell substrates, components of the medium used for cell culture, antibodies leaked from the column used in purification processes, and reagents used in other processes should be taken into account.

Currently, there is no official guideline in Japan focusing on the immunogenicity of therapeutic protein products. Regulatory requirements for immunogenicity assessment of therapeutic protein products vary case by case according to the nature of the products, their production process, and quality attributes (including impurities, stability) and intended clinical use, as well as patient-related factors.

This chapter describes (1) the impact of immunogenicity on safety and efficacy of therapeutic protein products, (2) the quality attributes and other factors that affect antibody formation against the products, and (3) approaches to assessing immunogenicity in nonclinical and clinical studies during drug development and in postmarketing surveillance. Based on these considerations together with Japan's unique experiences with the immunogenicity of therapeutic protein products, practical approaches to minimizing the risks associated with immunogenicity are discussed. The opinions presented here are personal.

4.2. IMMUNOGENICITY OF THERAPEUTIC PROTEIN PRODUCTS—IMPACT ON SAFETY AND EFFICACY

As shown in Figure 4.1, the drug substance of therapeutic protein products contains not only active ingredient but also impurities [12]. The active ingredient is composed of the desired product and product-related substances. Impurities consist of process-related and product-related substances. During storage, the active ingredient and product-related impurities may degrade, and most of these components can be immunogenic. Product-related substances and product-related impurities are molecular variants of the desired product, both of which have partially the same structure as the desired product; therefore, these components may contribute to the emergence of antibodies against the desired product. Moreover, impurities can act as adjuvants that may enhance the immunogenicity of the product [4]. As described in Sections 4.2.1 and 4.2.2, the consequences of antibody formation against the active ingredient and against the impurities are not the same.

4.2.1. Antibody Formation against Active Ingredient

When antibodies are formed against the active ingredient, the possible consequences are hypersensitivity reactions, change in the serum half-life of the

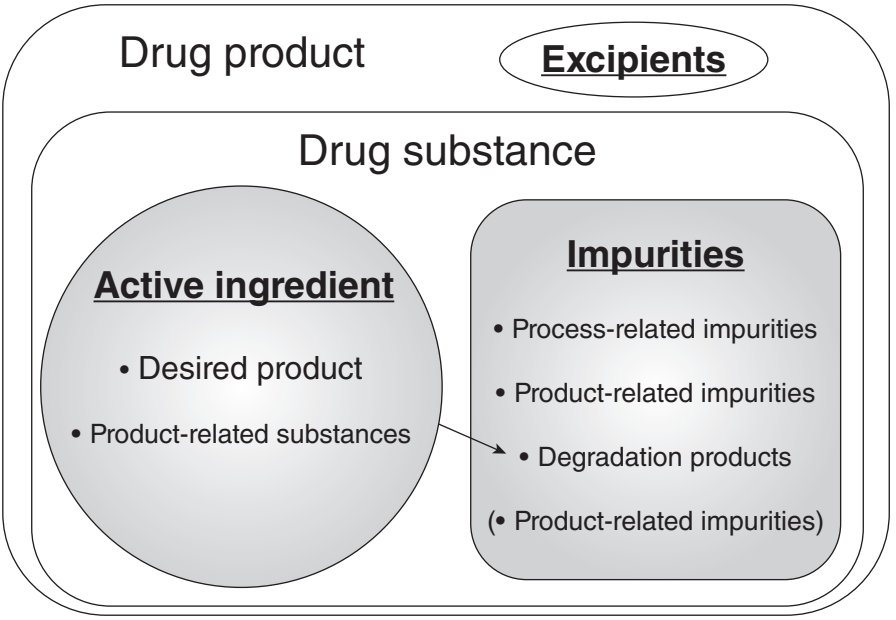

Fig. 4.1 Components of a therapeutic protein product. **Desired product** is the protein that has the expected structure, or the product that is expected from the DNA sequence and anticipated post-translational modification (including glycoforms) and from the intended downstream modification to produce an active biological molecule. **Product-related substances** are molecular variants of the desired product formed during manufacture and/or storage that are active and have no deleterious effect on the safety and efficacy of the drug product; these variants possess properties comparable to those of the desired product and are not considered impurities. **Product-related impurities** are molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety. **Process-related impurities** are impurities that are derived from the manufacturing process; they may be derived from cell substrates (e.g., host-cell proteins, host-cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables).

active ingredient, decrease in efficacy, or life-threatening adverse effects from neutralization of endogenous proteins by the anti-drug antibodies. The consequences after the formation of antibodies depend on the characteristics of the product and the features of the antibodies induced by the treatment.

If a product has xenogeneic amino acid sequences, the main concern is hypersensitivity reactions when the product is used repeatedly. For all kinds of therapeutic protein products, hypersensitivity reactions, changes in serum half-life, or decreased efficacy may occur. If a product has the same amino acid sequence as endogenous human proteins that have critical functions and work in nonredundant pathways, such as erythropoietin or thrombopoietin, severe

adverse effects could be caused by the emergence of neutralizing antibodies that cross-react with the endogenous proteins [13–15].

Among the antibody features that affect the clinical consequences of immunogenicity, neutralizing activity and immunoglobulin (Ig) class are especially important. Emergence of neutralizing antibodies may lead to a decrease in efficacy of the product. As previously mentioned, if the neutralizing antibodies react with endogenous proteins with critical functions, not only a decrease in efficacy but also life-threatening adverse effects would occur. In cases where IgE antibodies against the product components emerge, acute hypersensitivity reactions may be the main concern.

4.2.2. Antibody Formation against Process-Related Impurities

Since process-related impurities are nonhuman materials, potential immunogenicity is inevitable. Well-known process-related impurities that may provoke antibodies are host-cell-derived proteins [16], and the possible consequences of antibody formation are hypersensitivity reactions. Some patients already have antibodies against host-cell-derived proteins or components of cell culture medium, such as albumin, that might have been formed by exposure to microorganisms or food in daily life before beginning therapy. Therefore, even during the first treatment, hypersensitivity reactions caused by antibodies against process-related impurities need to be monitored. In some cases, potential hypersensitivity of patients against specific process-related impurities should be tested before clinical application of certain therapeutic protein products whose manufacturing process includes such reagents.

In addition to their own immunogenicity, process-related impurities can act as adjuvants for the active ingredient. These impurities may enhance antibody formation against the impurities per se or against other impurities. Therefore, to ensure the safety and efficacy of therapeutic protein products, extensive and efficient removal of such impurities should be especially important during product purification.

4.3. FACTORS AFFECTING THE IMMUNOGENICITY OF THERAPEUTIC PROTEIN PRODUCTS

Humoral immune responses are predominant in the immunogenesis of therapeutic protein products [17]. In general, antibodies, mainly IgM, are produced from B cells activated by the native conformational epitopes during initial exposure. When the B cells are activated by helper T cells that were activated by antigen-presenting cells that had taken up the antigens, immunoglobulin class switching occurs and the B cells then differentiate into antibody-producing plasma cells. In antigen-presenting cells, incorporated proteins are digested and the resulting peptides are presented on the cell surface by binding with

major histocompatibility antigen [MHC; human leukocyte antigen (HLA) refers to human MHC] class II molecules. It has been reported that the antibodies induced by therapeutic protein products are not only IgM but also IgG and IgE; therefore, mechanisms dependent on both B and T cells are involved in the antibody formation [17].

By genetic recombination of B-cell receptors or T-cell receptors, both B-cell and T-cell repertoires initially are highly variable in order to react with all kinds of exogenous materials [18]. As a consequence of the high variability of the receptors, the lymphocyte repertoire includes cells that react with self-antigens. The resulting self-reacting lymphocytes are deleted or induced to anergy after exposure to self-antigens via central and peripheral tolerance mechanisms [18]. However, in some situations such as onset of autoimmune disease or treatment by human protein products, self-tolerance is broken and self-reactive antibodies are produced. Considering the mechanisms of antibody formation, some features of the products such as presence of aggregates or coexistence with impurities may enhance the antibody formation pathways to overcome self-tolerance. For protein products carrying xenogeneic sequences, antibody formation is inevitable.

Factors affecting antibody formation against protein products include those discussed in the following subsections [19].

4.3.1. Product-Related Factors

4.3.1.1. Protein Primary Structure. If the protein product has xenogeneic or engineered sequences, these polypeptides are unavoidably immunogenic for humans. Mouse monoclonal antibodies, chimeric monoclonal antibodies, or engineered protein products are intrinsically immunogenic. Furthermore, even if the products have full human sequences, they can activate the human immune system. Since T-cell-mediated responses are involved in antibody formation against therapeutic protein products, human proteins containing peptide sequences that bind to HLA class II molecules with high affinity (namely, T-cell epitopes) might be highly susceptible to antibody formation [20]. On the other hand, recent evidence suggests that among T-cell epitopes certain kinds of peptides have an inhibitory activity on the immune response by activating regulatory T cells [21]. Therefore, each human protein has its own potential to induce or suppress antibody formation depending on its amino acid sequences.

4.3.1.2. Sugar Chain. Many therapeutic proteins are glycoproteins. In general, glycosylation reduces the immunogenicity of proteins [4]. This may be due in part to sugar chains masking conformational or peptide epitopes. On the other hand, when therapeutic protein products are produced using animal cells, the structures of sugar chains are different from those of human proteins [22]. Galactose-α-1,3-galactose (Gal (α1-3)Gal) and

N-glycolylneuraminic acid (NeuGc) are typical nonhuman glycans that can be present in glycoprotein products [22, 23]. These nonhuman glycans are known to be antigenic for humans [24, 25]. Gal(α1-3)Gal is a well-known antigen that is involved in rejection of organs transplanted from pigs to humans [24]. It is now recognized that all humans have IgG antibodies specific for Gal(α1-3)Gal [23]. In addition, it has been reported that most normal humans have anti-NeuGc antibodies [25]. These antibodies might have been raised by exposure to food materials or microorganisms containing these nonhuman glycans.

Obvious adverse events related to these nonhuman glycans have only recently been reported. In 2008, Chung et al. [23] reported a high prevalence of hypersensitivity reactions to cetuximab, a chimeric anti–epidermal growth factor receptor (anti-EGFR) antibody, caused by pre-existing IgE antibody against Gal(α1-3)Gal. Unlike most other monoclonal antibodies, cetuximab is produced in the mouse cell line SP2/0 that expresses α-1,3-galactosyltransferase. In addition, cetuximab has N-linked oligosaccharides in its variable regions. The presence of Gal(α1-3)Gal on both Fab segments of cetuximab might allow the efficient cross-linking of IgE receptors on mast cells. As mentioned previously, the patients who suffered hypersensitivity reactions had anti–Gal(α1-3)Gal IgE prior to therapy. The reason for the presence of IgE against Gal(α1-3)Gal is unclear. However, these cases alert us to the fact that we should learn more about the risks associated with antibodies specific for non-human glycans on protein products. To our knowledge, adverse events related to NeuGc associated with the use of biotechnology-derived protein products have not been reported, although NeuGc is known as Hanganutziu–Deicher (HD) antigen, which causes "serum sickness" reactions in humans treated with animal antiserum [22, 26–28].

4.3.1.3. Molecular Variants of the Desired Products. As shown in Figure 4.1, the drug substance of therapeutic protein products contains molecular variants of the desired product, that is, product-related substances and product-related impurities. These variants include aggregated, degraded, oxidized, or deamidated products. They arise during manufacture or storage. Among the variants, aggregates are well known to be immunogenic [29]. Since aggregates have repeated structure that efficiently activates B-cell receptors, they are liable to initiate immune responses [30]. In addition, aggregates enhance T-cell responses by activating antigen-presenting dendritic cells, thereby potentiating antibody formation [31]. Factors known to affect protein aggregation include protein concentration, pH, ionic strength, metal ions, organic solvent, temperature, pressure, shaking, shearing, interaction with the hydrophobic surface of containers, freeze-drying, reconstitution, and freeze–thaw cycles [32].

Even if the desired products have full human amino acid sequences, the molecular variants might constitute different epitopes from human endogenous proteins, and possibly be recognized as non-self-antigens. If the mechanisms of so-called epitope spreading are activated, antibodies against the desired product might subsequently be developed [33].

4.3.1.4. Impurities That Act as Adjuvants. Therapeutic protein products usually have small amounts of process-derived impurities such as host-cell proteins, host-cell DNA, cell culture medium components, column leachables, or processing reagents. These impurities may possibly work as adjuvants for the products. In the antibody-producing pathway, there are several steps that can be potentiated by adjuvants [34, 35]; therefore, impurities of different structure could possibly work independently or synergistically to enhance antibody formation. In the well-known case of pure red cell anemia associated with subcutaneous injection of the erythropoietin Eprex, leachates from the rubber syringe stopper used for drug products containing polysorbate 80 were suggested to have adjuvant activity for the surrogate antigen ovalbumin [36].

4.3.1.5. Formulation. Excipients such as human serum albumin and sugars may interact with the active ingredients and result in adducts that might constitute new epitopes. Inadequate formulation may lead to increased aggregates, thereby enhancing product immunogenicity.

4.3.1.6. Immunomodulatory Properties of the Product. The biological activities of therapeutic proteins also affect antibody formation. If a product has immunostimulatory or immunoinhibitory properties, formation of antibodies against the product might be stimulated or suppressed, respectively.

4.3.1.7. Route of Administration. Antibodies are formed more efficiently following subcutaneous or intramuscular injection than after intravenous injection. This may be because antigens are taken up more efficiently by antigen-presenting cells in subcutaneous or intramuscular tissues.

4.3.1.8. Storage Conditions and Product Handling. During storage, molecular variants of the desired product and product-related substances may increase. In addition, during handling by patients, improper storage may lead to deterioration of the sample [37].

4.3.2. Patient-Related Factors

4.3.2.1. Genetic Background (Genetic Defect, HLA Type). Patients congenitally lacking a gene do not have immunological tolerance to the corresponding gene product and therefore can readily generate antibodies to that product. In addition, it has been reported that the type of HLA can affect immune responses [38]. This is because the affinity of peptides, processed from proteins incorporated in antigen-presenting cells, with HLA depends on the structure of the HLA molecules.

4.3.2.2. Immune State of the Patient. Many diseases affect the immune state of the patient. In patients with a disease such as cancer that can compromise

immunological function, antibodies are less likely to be formed by treatment with protein products.

4.3.2.3. Concomitant Treatment. Concomitant treatments that affect immune responses can also affect antibody formation against protein products. When an immunosuppressant is concomitantly used in a patient's treatment, antibody formation against the products may decrease.

4.3.2.4. Previous Exposure to Similar Proteins. If patients have been exposed to the same protein product or other products with a similar structure, for example, a chimeric antibody product, antibodies can be formed upon subsequent administration.

4.3.2.5. Age. Different age group such as children or the elderly, may show different immune responses. Thus, data obtained from a particular age group cannot necessarily be extrapolated to other groups.

4.4. IMMUNOGENICITY ASSESSMENT REQUIRED IN DRUG DEVELOPMENT—CURRENT STATUS IN JAPAN

Currently there is no Japanese guideline focusing on immunogenicity issues of therapeutic protein products. However, in the review process for marketing authorization, immunogenicity problems are discussed sufficiently. In application dossiers for marketing authorization, applicants are requested to describe the rationale of the study protocol and the validity of the methods for antibody detection and characterization. Based on the data obtained, the safety and potential risks associated with the immunogenicity of the product need to be explained. The kind of data required for the assessment of immunogenicity depends on the nature and intended clinical use of the product. In some cases, safety assessment related to immunogenicity is continued in postmarketing surveillance.

4.4.1. Immunogenicity Tests in Nonclinical Studies

Human protein products are xenogeneic proteins for experimental animals, and antibodies arise with high frequency. Therefore, it is well recognized that immunogenicity tests in animals are generally not predictive of a potential for product immunogenicity in humans.

Nevertheless, nonclinical evaluation of antibody formation against the product in question is almost always performed. One of the principal purposes of antibody detection in nonclinical animal studies is to justify that the nonclinical studies have been conducted using relevant animal models with respect to immunological responses. Another is not necessarily to predict the immunogenicity of the product of interest in humans per se, but rather to investigate

the potential impact of the emergence of antibodies, if any, in humans on various safety- and efficacy-related parameters prior to a clinical study, as well as to support the interpretation of the nonclinical study results. Antibody responses should be characterized (e.g., titer, number of responding animals, neutralizing versus non-neutralizing), and their appearance should be correlated with any pharmacological and/or toxicological changes, where necessary and possible. Specifically, the effects of antibody formation on pharmacokinetic or pharmacodynamic parameters, the incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data [39].

There are several *in silico*, *in vitro*, and *in vivo* methods for estimating the relative immunogenicity of proteins in humans [40, 41]. These methods can be useful in the initial stages of drug development for engineered protein products in order to select a less immunogenic molecule as a new drug candidate.

In silico methods include identification of T-cell epitopes in the protein of interest that will be presented on antigen-presenting cells by binding to HLA class II molecules [20]. The number of T-cell epitope peptides and their affinity to HLA molecules are expected to correlate with immunogenicity of the protein. Recently, it was reported that some of the T-cell epitopes work as suppressors for antibody response by activating regulatory T cells [21]. Therefore, by *in silico* methods the immunogenic property of the protein is shown as a sum of T-cell epitopes that lead to the stimulation or suppression of the immune response. By *in vitro* T-cell activation assay, the potency of each T-cell epitope, identified by *in silico* experiments, to activate T cells can be evaluated. Correlation between *in silico* experimental results and clinical responses has been reported for several proteins [42]. Although there are several methods that predict the presence of B-cell epitopes, the quality of B-cell epitope predictions is considered to be too poor to be employed as a reliable tool [40].

In vivo methods using human protein transgenic mice and HLA transgenic mice have been reported [43, 44]. Since transgenic mice expressing human proteins are expected to be tolerant of the relevant therapeutic human proteins, breaking of self-tolerance by human protein products can be evaluated. In HLA transgenic mice, T-cell-mediated antibody formation that is activated by human-protein-derived peptides that bind to HLA molecules can be evaluated. Double transgenic mice expressing the human protein of interest and HLA would also be useful for evaluating the immunogenicity of human protein products. HLA transgenic mice can be used to confirm the results of *in silico* experiments by comparing the relative immunogenicity of two or more protein products carrying different amino acid sequences.

There is a possibility that *in vivo* methods may be used to evaluate comparability during the development of subsequent-entry protein products or to evaluate the consequences of a process change. In such cases, however, the

protein products to be tested have the same amino acid sequence; therefore, quality-related factors such as post-translational modifications of the product, product-related impurities, or process-related impurities would contribute to differences in immunogenicity of the products. Therefore, extensive physicochemical and biochemical analyses to reveal the quality attributes that may relate to immunogenicity would be more appropriate and more practical.

If immunogenicity tests in nonclinical studies are performed to predict immunogenicity in humans, it is necessary to thoroughly demonstrate the validity of the evaluation systems employed. However, since antibody formation depends not only on product-related factors but also on patient-related factors, we think that evaluation of immunogenicity in clinical studies is required for all new-entry protein products that will be administered repeatedly. Immunogenicity testing would be continued in postmarketing surveillance in addition to investigational clinical studies.

4.4.2. Immunogenicity Assessment in Clinical Studies and Postmarketing Surveillance

Adequate antibody detection and observation in clinical studies is most important for assessing the impact of antibody formation on safety and efficacy of the product. Immunogenicity assessment in clinical studies is necessary for all novel products except those used for a single administration. Even if other protein drug products with the same nonproprietary name have been approved, immunogenicity assessment may be necessary depending on the quality attributes of the drug product in question. This is because immunogenicity may depend on drug-product characteristics such as formulation as well as content and variety of product- and process-related impurities. For products that have been developed overseas, it is desirable to address any ethnic differences in the frequency of antibody formation.

In immunogenicity assessment using clinical samples, appropriateness of the antibody detection methods is the key to obtaining to reliable results. The tiered approach consisting of a screening assay, confirmatory assay, and neutralizing-antibody assay is the preferable strategy [19]. The requirements for each assay are different. For screening assays, relatively high throughput and certain detection of positive samples are required. The rationale of the criteria used for judging whether a sample is positive, for example, the cutoff value of optical density in an enzyme-linked immunosorbent assay (ELISA), should be shown. For confirmatory assays, confident judgment of the presence of antibodies is required. For neutralizing-antibody assays, methods that can adequately evaluate the biological activity of the drug should be used.

The methods commonly used for the detection of binding antibodies are ELISA, radioimmunoprecipitation (RIP) assay, electrochemiluminescent (ECL) assay, and surface plasmon resonance (SPR) assay. The advantages and disadvantages of these methods are listed in Table 4.2 [45]. For screening and confirmatory assays, ELISA and RIP would be the most commonly used

TABLE 4.2 Advantages and Disadvantages of Various Anti-Drug Antibody Assay Formats

Method	Advantage	Disadvantage
ELISA	Sensitive Inexpensive Equipment readily available	May not detect early immune response (especially rapidly dissociating or low-affinity antibodies) May be influenced by high levels of circulating drug (especially bridging format)
ECL	Sensitive Can be modified to respond in the presence of high levels of circulating drug	Equipment can be expensive May not easily detect rapidly dissociating antibodies
SPR	Method of choice for detecting early immune response Antibody characterization capabilities	Expensive equipment Generally less sensitive than RIP, ELISA, or ECL (but more sensitive for rapidly dissociating antibodies)
RIP	Sensitive Inexpensive Equipment readily available	May not detect early immune response May be influenced by high levels of circulating drug

Source: This table was adapted from "Assays and strategies for immunogenicity assessment" presented by Swanson SJ at the BMWP/BWP workshop on immunogenicity assessment of therapeutic proteins [45].

methods, respectively. SPR assays are useful for detecting an early immune response or characterizing antibody class. Other methods such as Western blotting can be used, if appropriate. However, there is no perfect method for antibody detection. Applicants should justify their strategies for antibody detection. It is desirable to measure not only IgG but also IgE and IgM antibodies in order to assess the possibility of hypersensitivity reactions and an early immune response, respectively. In the development of engineered protein products such as an insulin analogue, cross-reactivity of antibodies with the endogenous proteins (i.e., insulin in this case) should be investigated. If patients who received a therapy using protein products of similar structure have been found to have antibodies against the active ingredient before the study, antibody concentration or titer before the study can be used as a control for each case.

Regarding samples in which the presence of antibody was confirmed, neutralizing activity of the antibodies should be carefully examined, because the most distinctive clinical effect of anti-drug antibody formation is a decrease in efficacy of the product. Neutralizing antibodies that also inhibit the

functions of endogenous proteins can cause serious adverse reactions. The methods for quantifying neutralizing antibodies vary depending on the characteristics of the drugs, for example, enzyme activity assay for enzyme products, or virus-induced cytopathic effect assay for interferons. Appropriateness of the method employed should be described.

It is desirable for the antibody detection methods to be established and validated as quantitative methods with regard to specificity, precision, linearity, range, and limit of detection [46]. However, there are some difficulties in the establishment and validation of these methods, such as limited availability of positive control serum, and matrix effects due to high levels of circulating drug. Especially in early clinical studies, validation using positive control serum is difficult. The use of animal serum in which anti-drug antibodies are present is one possible approach. If international reference standards are available as a positive control, the assay could be validated using them. The influence of high levels of circulating drugs can be problematic, especially for protein products with long half-life such as recombinant monoclonal antibody drugs. Several pretreatment methods that can dissociate the antibodies from the drugs have been reported [47–49]. The appropriateness of such pretreatment methods should also be demonstrated.

Even if the investigational clinical studies were performed using an adequate study plan and validated antibody detection methods, immunogenicity-related risks for each product are not always fully revealed at the time of marketing authorization due to the limited number of patients enrolled in the clinical studies. Typical examples are the orphan drugs. In cases where further studies are necessary, risk assessment in postmarketing surveillance is also important. In the review documents of marketing authorization in Japan, we add declarations for some products that immunogenicity assessments are planned in postmarketing surveillance (e.g., lysosomal enzyme products, insulin analogue products, and interferons). Pharmacovigilance planning is reviewed before marketing authorization. In postmarketing surveillance, validation of the antibody detection method is still important. In some cases, improvement of the antibody detection method is requested by the regulatory agency at the time of marketing authorization approval. Thus, appropriate postmarketing surveillance in addition to the investigational clinical studies would reveal the immunogenicity risks of each product, regarding the frequency of emergence of binding or neutralizing antibodies and their clinical impact.

4.5. JAPANESE EXPERIENCE AND RISK-MINIMIZING APPROACH

4.5.1. Japanese Experience

The majority of protein products used in Japan is also approved overseas. Therefore, Japan and other regions might have shared similar experiences regarding immunogenicity concerns. Here we introduce two unique experi-

ences obtained in clinical studies conducted by Japanese companies. The products are recombinant human serum albumin (rHSA) and adalimumab, a recombinant human anti-TNF-α antibody. Since Eprex has not been approved in Japan, there was no accumulation of reports of the obvious adverse event of pure red cell aplasia around 2001.

Recombinant HSA produced by the yeast *Pichia pastoris* is a protein product developed by a Japanese company [50, 51]. To our knowledge it is the only product that contains rHSA as an active ingredient. It took 10 years from application to approval of the product to confirm the quality, safety, and efficacy of the product. Because the dose of rHSA, 12.5 g, is much higher than other products, the content of process-derived impurities should be reduced to as low a level as possible to ensure safety. As in the case of nonhuman glycans, some people have anti-yeast IgE before treatment due to exposure to yeast components in daily life. In the clinical study of rHSA, a challenge study was performed in anti-pichia IgE–positive healthy volunteers. Unfortunately, severe hypersensitivity reactions occurred in two of four volunteers. After that, the manufacturing process was further improved to lower the content of host-cell proteins. Then the usefulness and potential risks of the product, including hypersensitivity reactions caused by anti-pichia IgE, were carefully reviewed. In 2008, rHSA was approved. To avoid hypersensitivity reactions caused by pre-existing anti-pichia IgE, serum tests for the presence of anti-pichia IgE were made mandatory before treatment.

Adalimumab is an example that illustrates an ethnic difference in the immunogenicity of a therapeutic protein product [52]. When clinical studies of adalimumab were conducted in Japan, there had already been clinical data available overseas. When adalimumab was administered at a dose of 20 mg biweekly, the ratio of anti-adalimumab antibody (AAA)–positive patients was 40.2% in Japan compared with 17.9% in Western countries (Table 4.3). Similar results were obtained with other doses. Therefore, the incidence of AAA-positive patients was about two times higher in Japanese than in Westerners. The observed AAAs were anti-idiotype antibodies. In AAA-positive patients, the clearance of adalimumab was faster and the efficacy was lower than in antibody-negative patients.

TABLE 4.3 Incidence of Antibody-Positive Patients in the Clinical Studies of Adalimumab

	Incidence of Antibody-Positive Patients	
Treatment	Japan (M02–575 Study)	Overseas (DE011 Study)
20 mg biweekly	40.2% (35/87)	17.9% (19/106)
20 mg weekly	–	9.8% (11/112)
40 mg biweekly	44.0% (40/91)	17.7% (20/113)
40 mg weekly	–	3.9% (4/103)
80 mg biweekly	26.4% (23/87)	–

In the Pharmaceuticals and Medical Devices Agency (PMDA) review report for marketing authorization, the reasons for the high frequency of AAAs in Japan are explained by the applicant as follows: adalimumab is a fully human antibody; therefore, it was recognized as a human antibody by the endogenous immune system and was incorporated in the anti-idiotype network, thereby resulting in the high frequency of emergence of anti-idiotype antibodies against adalimumab. The bases of this explanation are as follows: (1) Adalimumab has the amino acid sequence of allotype $GM1^{z,a}$, and most (84%) Japanese have $GM1^{z,a}$ allotype, whereas this allotype is less frequent (29%) in Westerners. (2) The observed antibodies were anti-idiotype antibodies.

We think this explanation is just one of the possible hypotheses that could account for these observations. However, although it is recognized that human antibody products still might be immunogenic in humans, the mechanism of antibody formation might be different from that for other kinds of antibodies.

4.5.2. Risk-Minimizing Approach

Possible risk factors related to immunogenicity are shown in Table 4.4. Basically, there are two risk-minimizing approaches. The first approach, which is a pre-

TABLE 4.4 Risk Factors Related to Immunogenicity

Categories	Risk Factors
Product-Related	
Protein primary structure	Xenogeneic sequences Effector T-cell epitopes Same sequence as endogenous protein with unique and critical functions
Post-translational modification	Nonhuman glycans
Biological activity	Immunostimulatory function
Purity	Product-related impurities Process-related impurities
Route	Subcutaneous Intramuscular
Administration	Repeat
Patient-Related	
Genetic background	Genetic defect of the corresponding gene Specified HLA type
Immune state	Not suppressed
Pre-existing antibody against process-related impurities	Present
Previous treatment	Treatment with similar protein products
Concomitant treatment	Immunostimulatory drugs

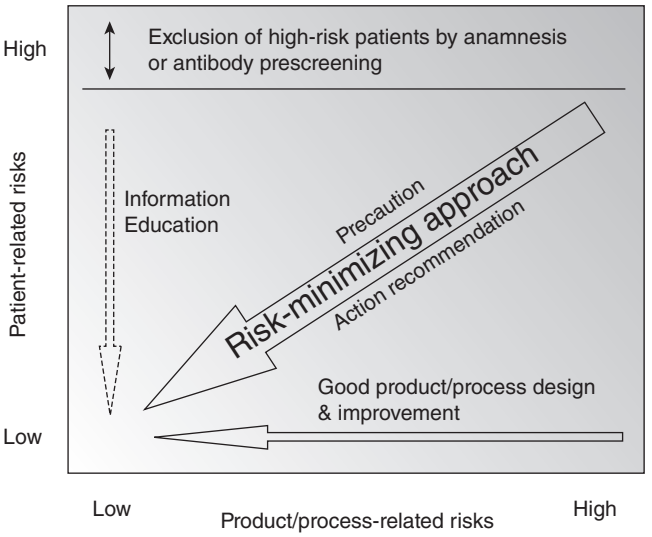

Fig. 4.2 Approaches for minimizing risks related to immunogenicity. (This figure was drawn with reference to the framework of the presentation of J. Ramsbotham, Solvay Pharm. NL/EFPIA.)

requisite, is to reduce product-related and process-related risks as much as possible, whenever they are already known or predicted. This includes good product and process design and improvement along with drug development, which should be carried out and justified primarily by the manufacturers. The second approach is to manage treatment with the known risks in mind. So, the critical step is to identify both product- and process-related risks specific to each case. Figure 4.2 illustrates this risk-minimizing approach.

4.5.2.1. Reducing Product-Related Risk Factors. In the initial stages of drug development, if there are several candidates with different amino acid sequences available for selection, the least immunogenic sequence based on *in silico* experiments would be the preferable choice. For any kind of product, the manufacturing process and formulation should be optimized to reduce process- and product-related impurities that are known to affect immunogenicity, for example, host-cell proteins and aggregates. Selecting the route of administration may also contribute to reducing the risk of immunogenicity, if appropriate.

4.5.2.2. Management of Treatment with the Known Risks in Mind. Even if product- and process-related risk factors are minimized by good product and process design and improvement during drug development, residual risks still exist. In addition, patient-related risks are difficult for the manufacturers to manage. One of the possible approaches for minimizing patient-related risks

is to exclude high-risk patients by antibody prescreening tests or by using the anamnesis of each patient. The case of rHSA is a good example of this approach. Administration of a product to those patients who have experienced hypersensitivity reactions to that product or other products containing similar active ingredients is considered to be a contraindication for almost all products.

Another possible approach is for the product label to state the precautions to be taken. In Japan, for protein products for which immunogenicity-related risks are a concern, the precautions and recommended actions to be taken are written on the product label (Table 4.5). During the review for marketing authorization approval, the precautions and recommended actions to be taken are decided upon by a discussion based on the clinical data. The aforementioned contraindication and the necessity of IgE prescreening for rHSA are examples of precautions written on product labels (Table 4.5A,B). As shown in Table 4.5C, recommended actions are provided on the label together with the known risks of the product. For example, in the case of lysosomal enzyme products such as agalsidase beta used for enzyme replacement therapy in which anti-drug IgG was detected at a high frequency in clinical studies, periodic tests for anti-drug IgG are recommended. For products for which the emergence of neutralizing antibodies is a concern, for example, interferon beta-1a, neutralizing-antibody assays are recommended in the cases where efficacy is insufficient or decreases after repeated administration. For products containing xenogeneic amino acid sequences, precautions about the emergence of hypersensitivity reactions after repeated administration are written on the product label. In the case of patients who have been treated with similar therapeutic protein products, for example, mouse monoclonal antibodies or chimeric antibodies, it is stated on the product label that such patients should be screened for the presence of pre-existing antibodies such as human anti-mouse antibody (HAMA) or human anti-chimeric antibody (HACA).

In addition to written notices on product labels, education of physicians and patients is also important. The first purpose is to ensure that the precautions written on the product label are implemented. Providing information to physicians about possible clinical symptoms caused by the emergence of antibodies will help reduce such risks. The other purpose is to ensure the proper handling of products, because inadequate handling of products such as storage under inappropriate conditions may potentiate immunogenicity-related risks by increasing product-related impurities or leachate from containers.

4.6. CONCLUDING REMARKS

Experience gained from the use of therapeutic protein products has shown that concern about immunogenicity cannot be fully eliminated. However, due to their specific efficacy in various diseases, therapeutic protein products are indispensable therapeutic agents the use of which cannot be substituted by the

TABLE 4.5 Examples of Immunogenicity-Related Precautions Written in Product Labels Approved in Japan

(A) Contraindication

Product	Contraindication
Almost all products	Patients who have anamnesis of hypersensitivity reactions to components of the product or other products containing similar active ingredient

(B) Mandatory Prescreening

Product	Precaution	Obligation
Human Serum Albumin	Hypersensitivity reactions can occur in patients who have anti-pichia antibody.	Anti-pichia IgE prescreening

(C) Recommended Actions

Product	Precaution	Recommended Action
Agalsidase Beta Laronidase Alglucosidase Alfa Idursulfase Galsulfase	Anti-drug IgG will emerge with high frequency. Infusion reactions can be caused by the IgG.	Periodic IgG testing
Mecasermin	Anti-drug antibody can be induced by continuous treatment.	Antibody prescreening by skin prick test to predict hypersensitivity reactions Discontinuation of treatment if decrease in efficacy is observed with emergence of antibody
Octocog Alfa Rurioctocog Alfa	Attention should be paid to the possible emergence of inhibitors.	Inhibitor test if decrease in efficacy is observed
Epoetin Alfa Epoetin Beta Darbepoetin Alfa	Pure red cell aplasia (PRCA) can be caused by anti-erythropoeitin antibody.	Discontinuation of treatment if PRCA is observed
Somatropin	Anti-drug antibody can be induced by continuous treatment.	Discontinuation of treatment if decrease in efficacy is observed with emergence of antibody
Interferon Beta-1a	Neutralizing antibodies can emerge.	Test for neutralizing antibodies if efficacy is insufficient after 1 year of treatment

use of small-molecule chemical drugs. It should also be noted that immunogenicity-related risk factors may be specific to the individual therapeutic protein products and its intended clinical use. Taken together, it is critical to minimize potential adverse immunogenic consequences in the clinical use of products of interest by a case-by-case approach. In the future, designing or selecting less immunogenic molecules will be more important and feasible based on the recent progress in *in silico* technology for estimating the immunogenicity of proteins. It will be desirable to have relevant animal models validated to reliably predict the potential of a protein to induce immunogenic responses in humans, and long-term clinical studies enrolling a large number of patients at a preapproval stage. However, at least in the near future due to technical limitations, data from preapproval nonclinical and clinical studies may be insufficient to identify all immunogenic safety profiles. Therefore, safety with respect to immunogenicity can only be assessed through (long-term well-designed) postapproval surveillance programs.

In summary, immunogenicity-related risks of therapeutic protein products may be minimized by reducing the immunogenicity-related impurities to as low a level as possible, identifying the risk factors for individual product and patients, excluding high-risk patients by antibody prescreening or anamnesis, monitoring antibodies by appropriate tests periodically or in a timely manner, and ensuring the implementation of written precautions and recommendations on product labels.

REFERENCES

1. Rosenberg, A.S., Worobec, A. (2004). A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 2. Considering host-specific and product-specific factors impacting immunogenicity. *BioPharm Int*, Dec. 1, 34–42.
2. Rosenberg, A.S., Worobec, A. (2005). A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 3. Effects of manufacturing changes in immunogenicity and the utility of animal immunogenicity studies. *BioPharm Int*, Jan. 1.
3. Worobec, A., Rosenberg, A.S. (2004). A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 1. Considering consequences of the immune response to a protein. *BioPharm Int*, *Nov. 1*, 22–26.
4. Koren, E., Zuckerman, L.A., Mire-Sluis, A.R. (2002). Immune responses to therapeutic proteins in humans—clinical significance, assessment and prediction. *Curr Pharm Biotechnol*, *3*, 349–360.
5. Du, X., Tang, J.G. (1998). Effects of deleting A19 tyrosine from insulin. *Biochem Mol Biol Int*, *44*, 507–513.
6. Heding, L.G., Marshall, M.O., Persson, B., Dahlquist, G., Thalme, B., Lindgren, F., Akerblom, H.K., Rilva, A., Knip, M., Ludvigsson, J., et al. (1984). Immunogenicity of monocomponent human and porcine insulin in newly diagnosed type 1 (insulin-dependent) diabetic children. *Diabetologia*, *27* (Suppl.), 96–98.

7. Porter, S. (2001). Human immune response to recombinant human proteins. *J Pharm Sci*, *90*, 1–11.
8. Abdul-Ahad, A.K., Galazka, A.R., Revel, M., Biffoni, M., Borden, E.C. (1997). Incidence of antibodies to interferon-beta in patients treated with recombinant human interferon-beta 1a from mammalian cells. *Cytokines Cell Mol Ther*, *3*, 27–32.
9. Larocca, A.P., Leung, S.C., Marcus, S.G., Colby, C.B., Borden, E.C. (1989). Evaluation of neutralizing antibodies in patients treated with recombinant interferon-beta ser. *J Interferon Res*, *9* (Suppl. 1), S51–S60.
10. Perini, P., Facchinetti, A., Bulian, P., Massaro, A.R., Pascalis, D.D., Bertolotto, A., Biasi, G., Gallo, P. (2001). Interferon-beta (INF-beta) antibodies in interferon-beta1a- and interferon-beta1b-treated multiple sclerosis patients. Prevalence, kinetics, cross-reactivity, and factors enhancing interferon-beta immunogenicity in vivo. *European Cytokine Network*, *12*, 56–61.
11. van de Weert, M., Moller, E.H. (2008). Immunogenicity of biopharmaceuticals: causes, methods to reduce immunogenicity, and biosimilars. In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Moller EH, eds. Springer, New York, pp. 97–111.
12. Specifications: test procedures and acceptance criteria for biotechnological/biological products (ICH Q6B guideline). (1999). Available at http://www.pmda.go.jp/ich/q/q6b_01_5_1e.pdf
13. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New Engl J Med*, *346*, 469–475.
14. Chamberlain, P., Mire-Sluis, A.R. (2003). An overview of scientific and regulatory issues for the immunogenicity of biological products. *Dev Biol*, *112*, 3–11.
15. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
16. Pavlovic, M., Girardin, E., Kapetanovic, L., Ho, K., Trouvin, J.H. (2008). Similar biological medicinal products containing recombinant human growth hormone: European regulation. *Hormone Res*, *69*, 14–21.
17. De Groot, A.S., Moise, L. (2007). Prediction of immunogenicity for therapeutic proteins: state of the art. *Curr Opin Drug Discov Dev*, *10*, 332–340.
18. Foged, C., Sundblad, A. (2008). Immune reactions towards biopharmaceuticals—a general, mechanistic overview. In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Moller EH, eds. Springer, New York, pp. 1–25.
19. European Medicines Agency (EMEA). (2007). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins (EMEA/CHMP/BMWP/14327/2006). Available at http://www.emea.europa.eu/pdfs/human/biosimilar/1432706enfin.pdf
20. De Groot, A.S., McMurry, J., Moise, L. (2008). Prediction of immunogenicity: in silico paradigms, ex vivo and in vivo correlates. *Curr Opin Pharmacol*, *8*, 620–626.
21. De Groot, A.S., Moise, L., McMurry, J.A., Wambre, E., Van Overtvelt, L., Moingeon, P., Scott, D.W., Martin, W. (2008). Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes." *Blood*, *112*, 3303–3311.

22. Dingermann, T. (2008). Recombinant therapeutic proteins: production platforms and challenges. *Biotechnol J*, *3*, 90–97.

23. Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., Murphy, B.A., Satinover, S.M., Hosen, J., Mauro, D., Slebos, R.J., Zhou, Q., Gold, D., Hatley, T., Hicklin, D.J., Platts-Mills, T.A. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *New Engl J Med*, *358*, 1109–1117.

24. McKenzie, I.F., Xing, P.X., Vaughan, H.A., Prenzoska, J., Dabkowski, P.L., Sandrin, M.S. (1994). Distribution of the major xenoantigen (Gal(α1–3)Gal) for pig to human xenografts. *Transplant Immunol*, *2*, 81–86.

25. Tangvoranuntakul, P., Gagneux, P., Diaz, S., Bardor, M., Varki, N., Varki, A., Muchmore, E. (2003). Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc Natl Acad Sci USA*, *100*, 12045–12050.

26. Higashi, H., Naiki, M., Matuo, S., Okouchi, K. (1977). Antigen of "serum sickness" type of heterophile antibodies in human sera: identification as gangliosides with *N*-glycolylneuraminic acid. *Biochem Biophys Res Commun*, *79*, 388–395.

27. Kasukawa, R., Kano, K., Bloom, M.L., Milgrom, F. (1976). Heterophile antibodies in pathologic human sera resembling antibodies stimulated by foreign species sera. *Clin Exp Immunol*, *25*, 122–132.

28. Merrick, J.M., Zadarlik, K., Milgrom, F. (1978). Characterization of the Hanganutziu–Deicher (serum-sickness) antigen as gangliosides containing *N*-glycolylneuraminic acid. *Int Arch Allergy Appl Immunol*, *57*, 477–480.

29. Rosenberg, A.S. (2006). Effects of protein aggregates: an immunologic perspective. *AAPS J*, *8*, E501–E507.

30. Kessler, M., Goldsmith, D., Schellekens, H. (2006). Immunogenicity of biopharmaceuticals. *Nephrol Dial Transpl*, *21* (Suppl. 5), v9–v12.

31. Bachmann, M.F., Dyer, M.R. (2004). Therapeutic vaccination for chronic diseases: a new class of drugs in sight. *Nature Rev*, *3*, 81–88.

32. Wang, W. (2005). Protein aggregation and its inhibition in biopharmaceutics. *Int J Pharm*, *289*, 1–30.

33. Barbosa, M.D., Celis, E. (2007). Immunogenicity of protein therapeutics and the interplay between tolerance and antibody responses. *Drug Discov Today*, *12*, 674–681.

34. Schijns, V.E. (2000). Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol*, *12*, 456–463.

35. Storni, T., Kundig, T.M., Senti, G., Johansen, P. (2005). Immunity in response to particulate antigen-delivery systems. *Adv Drug Deliv Rev*, *57*, 333–355.

36. Ryan, M.H., Heavner, G.A., Brigham-Burke, M., McMahon, F., Shanahan, M.F., Gunturi, S.R., Sharma, B., Farrell, F.X. (2006). An in vivo model to assess factors that may stimulate the generation of an immune reaction to erythropoietin. *Int Immunopharmacol*, *6*, 647–655.

37. Sharma, B. (2007). Immunogenicity of therapeutic proteins. Part 1. Impact of product handling. *Biotechnol Adv*, *25*, 310–317.

38. Barbosa, M.D., Vielmetter, J., Chu, S., Smith, D.D., Jacinto, J. (2006). Clinical link between MHC class II haplotype and interferon-beta (IFN-beta) immunogenicity. *Clin Immunol*, *118*, 42–50.

39. Preclinical safety evaluation of biotechnology-derived pharmaceuticals (ICH S6 guideline). (1997). Available at http://www.pmda.go.jp/ich/s/s6_00_2_22e.pdf
40. Roggen, E.L. (2008). Models for prediction of immunogenicity. In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Moller EH, eds. Springer, New York, pp. 75–95.
41. Stas, P., Lasters, I. (2009). Strategies for preclinical immunogenicity assessment of protein therapeutics. *IDrugs*, *12*, 169–173.
42. Koren, E., De Groot, A.S., Jawa, V., Beck, K.D., Boone, T., Rivera, D., Li, L., Mytych, D., Koscec, M., Weeraratne, D., Swanson, S., Martin, W. (2007). Clinical validation of the "in silico" prediction of immunogenicity of a human recombinant therapeutic protein. *Clin Immunol*, *124*, 26–32.
43. Depil, S., Angyalosi, G., Morales, O., Delacre, M., Delhem, N., Francois, V., Georges, B., Hammer, J., Maillere, B., Auriault, C., Pancre, V. (2006). Peptide-binding assays and HLA II transgenic Abeta degrees mice are consistent and complementary tools for identifying HLA II–restricted peptides. *Vaccine*, *24*, 2225–2229.
44. Palleroni, A.V., Aglione, A., Labow, M., Brunda, M.J., Pestka, S., Sinigaglia, F., Garotta, G., Alsenz, J., Braun, A. (1997). Interferon immunogenicity: preclinical evaluation of interferon-alpha 2a. *J Interferon Cytokine Res*, *17* (Suppl. 1), S23-S27.
45. Swanson, S.J. (2007). Assays and strategies for immunogenicity assessment. Presented at BMWP/BWP workshop on immunogenicity assessment of therapeutic proteins. Available at http://www.emea.europa.eu/pdfs/conferenceflyers/bmwp/swanson.pdf
46. Validation of analytical procedures: text and methodology (ICH Q2 guideline). (1994). Available at http://www.pmda.go.jp/ich/q/q2r2_97_10_28e.pdf
47. Bourdage, J.S., Cook, C.A., Farrington, D.L., Chain, J.S., Konrad, R.J. (2007). An affinity capture elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. *J Immunol Methods*, *327*, 10–17.
48. Patton, A., Mullenix, M.C., Swanson, S.J., Koren, E. (2005). An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J Immunol Methods*, *304*, 189–195.
49. Smith, H.W., Butterfield, A., Sun, D. (2007). Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA. *Regul Toxicol Pharmacol*, *49*, 230–237.
50. Pharmaceuticals and Medical Devices Agency (PMDA). (2007). Human serum albumin (genetical recombination); PMDA approval report (in Japanese). Available at http://www.info.pmda.go.jp/shinyaku/g071007/400315000_21900AMZ00083_Q101_1.pdf
51. Kobayashi, K. (2006). Summary of recombinant human serum albumin development. *Biologicals*, *34*, 55–59.
52. Pharmaceuticals and Medical Devices Agency (PMDA). (2008). Adalimumab (genetical recombination); approval report from PMDA (in Japanese). Available at http://www.info.pmda.go.jp/shinyaku/g080405/10015900_22000AMX01598_A101_1.pdf

PART III

PRINCIPAL TECHNOLOGIES EMPLOYED FOR THE QUANTIFICATION OF ANTI-DRUG ANTIBODIES

5

ENZYME IMMUNOASSAYS AND RADIOIMMUNOASSAYS FOR QUANTIFICATION OF ANTI-TNF BIOPHARMACEUTICALS AND ANTI-DRUG ANTIBODIES

Klaus Bendtzen and Morten Svenson

5.1. INTRODUCTION

With the discovery of the central role of tumor necrosis factor alpha (TNF) in the pathogenesis of many immunoinflammatory diseases, specific inhibition of the activities of this pleiotropic cytokine has revolutionized the treatment of patients with rheumatoid arthritis (RA), juvenile idiopathic arthritis, ankylosing spondylitis (Bechterew's disease), inflammatory bowel diseases (Crohn's disease and ulcerative colitis), severe psoriasis, chronic uveitis, sarcoidosis, Wegener's granulomatosis, and others [1–7]. The first of a range of human IgG-derived constructs targeting TNF, infliximab (Remicade, Centocor Ortho Biotech), has been in clinical use since the mid-1990s, but several other antibody constructs have later emerged, including so-called humanized and fully human monoclonal antibodies (mAbs), Fab′ fragments of mAbs, and a TNF receptor construct (Fig. 5.1).

All these TNF inhibitors may dramatically lower disease activity and, in some patients, induce remission. Unfortunately, however, not all patients respond favorably. For example, patients suffering from Crohn's disease do

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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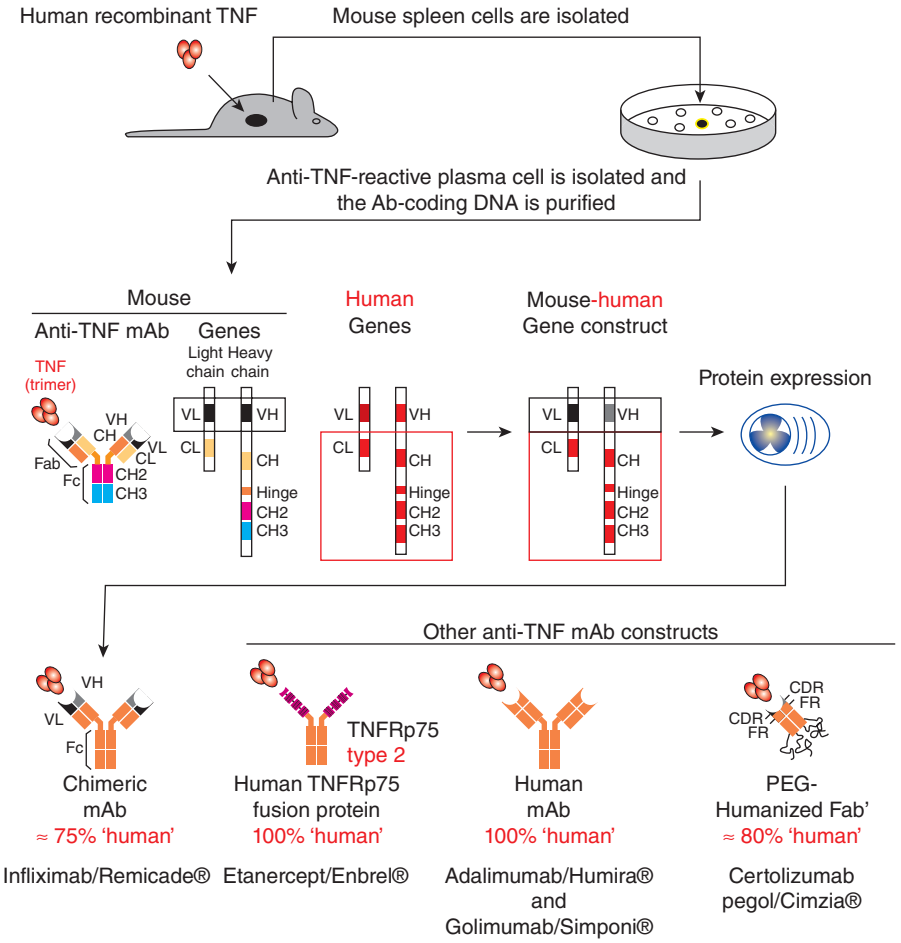

Fig. 5.1 Genetically engineered anti-TNF immunoglobulin constructs. Principle behind construction of infliximab (Remicade) is shown along with the structures of subsequently marketed TNF inhibitors. Anti-TNF-reactive splenocytes from mice immunized with human TNF are isolated and the light- and heavy-chain genes (VL and VH segments) are spliced together with human IgG1 genes (CL, CH, Hinge, CH2, and CH3 segments) to form the chimeric light and heavy chains. The antibody is produced when these constructs are expressed in antibody-secreting immortalized myeloma cells. (Abbreviations: VL and VH, variable regions of IgG on light and heavy chains, respectively; CL, CH, CH2, and CH3, constant regions of IgG on light and heavy chains, respectively; Fab′, fragment antigen binding, including the variable parts of IgG; Fc, human IgG1 Fc region; CDR, complementarity-determining regions; FR, framework regions; mAb, monoclonal antibody; PEG, polyethylene glycol; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.) (See color insert.)

not benefit from etanercept, and some patients treated with currently approved anti-TNF constructs either do not respond at all (primary response failure) or they respond initially but have later relapses (secondary response failure) despite increased dosage and/or more frequent administration of the drugs [8]. The mechanism(s) underlying these response failures are not entirely clear and the problem has received little attention until recently. This is surprising since drug delivery resembles effective vaccination procedures, that is, repeated and in most cases subcutaneous administration of non-self-proteins. Immunogenicity is in fact a potential feature of all biological agents, and antibody-based biopharmaceuticals are no exception in that they trigger both B and T lymphocytes, which may cause both immediate and delayed hypersensitivity reactions and limit therapeutic efficacy [9, 10].

Screening for anti-drug antibodies (ADAs) in clinical trials, now a regulatory requirement [11, 12], has also drawn attention to other issues of antibody therapies, including substantial interindividual and even intraindividual variations in bioavailability and pharmacokinetics [10, 13, 14]. Recognition of the benefits of "individualized or personalized medicine" is therefore emerging, which in this context means optimizing therapies according to individual needs rather than using standardized regimens deducted from a few pivotal trials in cohorts of patients of both sexes and with substantial variations in age, comorbidities, and concurrent therapies [8].

Considering the aforementioned problems and the considerable cost of antibody therapies, monitoring patients for circulating levels of drugs and ADAs is warranted so that treatment can be tailored individually, providing effective yet safe and economical long-term therapies. Unfortunately, standard laboratory technologies, usually in the form of solid-phase enzyme-linked immunosorbent assays (ELISAs), may not provide sufficiently useful data in the clinical setting, as is the case with other chronic biotherapies [15]. These methods are often subject to matrix effects when used to test serum or plasma samples, and blood components such as rheumatoid factors, antiallotypic antibodies (in previously immunized patients), and complement factors ($C1qr_2s_2$) may interfere with readouts and jeopardize clinical decision making [10, 16–18].

5.2. ANTI-TNF BIOPHARMACEUTICALS

Figure 5.1 shows the five anti-TNF antibody constructs currently in clinical use [19]. Infliximab (Remicade) is a mouse–human immunoglobulin IgG1-κ anti-TNF mAb, adalimumab (Humira, Abbott) is a fully human IgG1-κ anti-TNF mAb, and certolizumab pegol (Cimzia, UCB, Inc.) is a pegylated Fab′ fragment of a humanized anti-TNF mAb where the complementarity-derived variable regions from a mouse mAb against human TNF have been grafted into human IgG1 Fab′. Another TNF inhibitor, etanercept (Enbrel, Amgen), is a fusion protein of the human type 2 TNF receptor and human IgG1. The recently

developed golimumab (Simponi, Centocor Ortho Biotech) is a fully human IgG1-κ anti-TNF mAb approved for treatment of patients with RA, psoriatic arthritis, and ankylosing spondylitis [20].

All the aforementioned anti-TNF antibody constructs target both the soluble and membrane-associated forms of TNF, thus inhibiting TNF from triggering cellular TNF receptors. In addition, infliximab and adalimumab and, possibly, golimumab may have the potential to kill cells carrying membrane-bound TNF because of complement activation and/or through binding to Fc-γ receptors and antibody-dependent cell cytotoxicity.

5.3. ASSAYS FOR DRUG BIOAVAILABILITY, PHARMACOKINETICS, AND IMMUNOGENICITY

Monitoring bioavailability and pharmacokinetics also appears rational when one considers the inherent variability in the activity of chronic inflammatory diseases amenable to anti-TNF therapies. Monitoring is further underscored by the significant interindividual and even intraindividual variations that would be expected when "large molecules" are administered subcutaneously (the preferred route of administration of anti-TNF therapies), in many cases by the patients themselves [8]. It is therefore not a surprise that a drug concentration–effect relationship has consistently been reported in anti-TNF-treated patients, notably in RA and Crohn's disease [13, 21–25]. For example, high serum levels of infliximab just before an infusion, that is, high trough levels, have been associated with substantial clinical improvement, whereas low trough levels are associated with poor clinical response [13, 21].

5.3.1. Assays for TNF Inhibitors

Several different methods have been used to assess circulating levels of TNF inhibitors. Most are based on ELISA formats using either specific antibodies to the drugs, as both capture and detection antibodies, or tagged anti-human IgG antibodies to detect drugs bound to TNF coated to plastic wells or beads (Fig. 5.2). These assays are relatively simple but have shortcomings related to matrix effects and interference by rheumatoid factors, complement components, and other serum factors. It should also be noted that solid-phase assays in general are artifactual and do not mimic the clinical situation very closely due to the introduction of aggregated and/or denatured reactants.

5.3.1.1. Fluid-Phase Radioimmunoassay. The test principle of this radioimmunoassay (RIA) has been detailed previously and is summarized in Figure 5.3 [13, 16, 25]. While the use of radioactivity prevents broad application, this assay has advantages compared with most ELISAs. It is functional in that it shows the capacity of the drug to bind TNF rather than disclosing (epitopes on) a protein that may or may not be functional (see Fig. 5.3, left). Our RIA

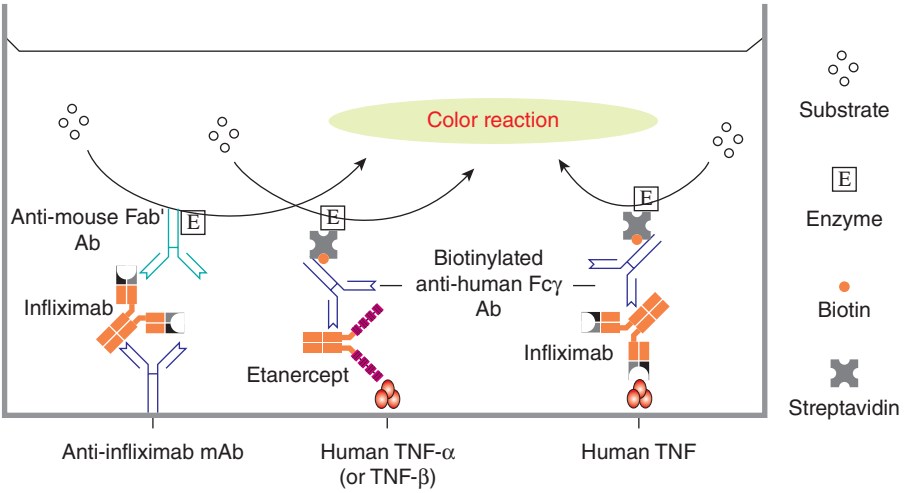

Fig. 5.2 Examples of ELISA formats for quantification of therapeutic anti-TNF antibody constructs. (See color insert.)

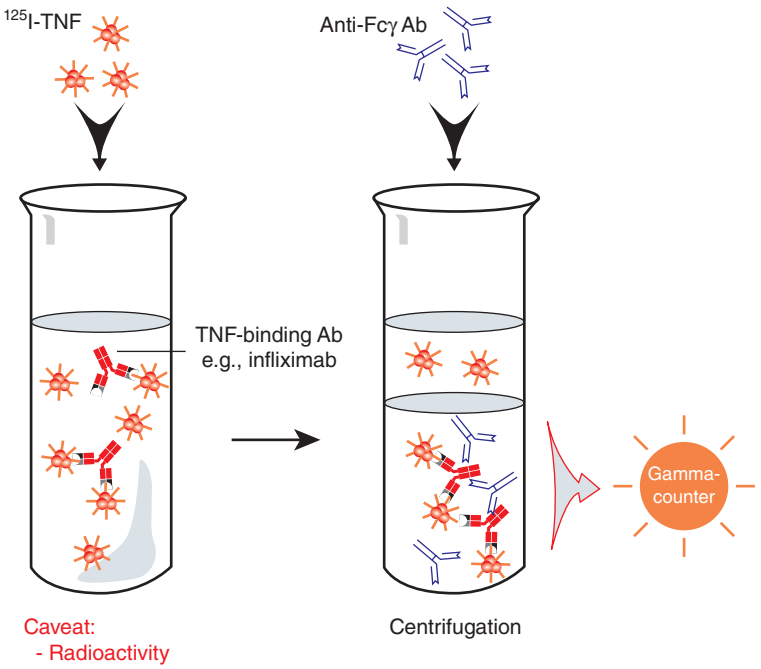

Fig. 5.3 Fluid-phase RIA for ^{125}I-TNF-binding capacity [10, 13, 16]. In this example, patient serum containing infliximab is incubated with ^{125}I-TNF followed by addition of rabbit anti-human IgG-Fc antibody. Bound and free ^{125}I-TNF are separated by centrifugation, and the pelleted radioactivity is counted and compared with a similar setup using the same amount of normal serum and known amounts of infliximab or any other TNF-binding whole antibody. (See color insert.)

is also a fluid-phase assay, which is less likely to yield false-positive results due to nonspecific binding or false-negative results due to epitope masking.

The RIA has been used to monitor infliximab-, adalimumab-, and etanercept-treated patients suffering from various chronic immunoinflammatory diseases, including RA and Crohn's disease [10, 13, 16, 25, 26]. An example is shown in Figure 5.4. Infliximab trough levels in serum of patients with RA varied considerably after intravenous infusions of a standard dosage of infliximab, both initially and after the appearance of ADAs, as discussed later. Interestingly, pronounced baseline disease activity was associated with low early infliximab levels, possibly because these patients are in need of more TNF inhibitor than the average patient deducted from pivotal trials (Fig. 5.4).

Recent data also show that low serum trough levels of infliximab correlate with poor clinical response in patients with Crohn's disease [26]. Thus, 7 out of 8 patients (88%) who had secondary loss of response to infliximab had serum trough levels below 0.5 µg/mL of the drug, whereas only 2 out of 19

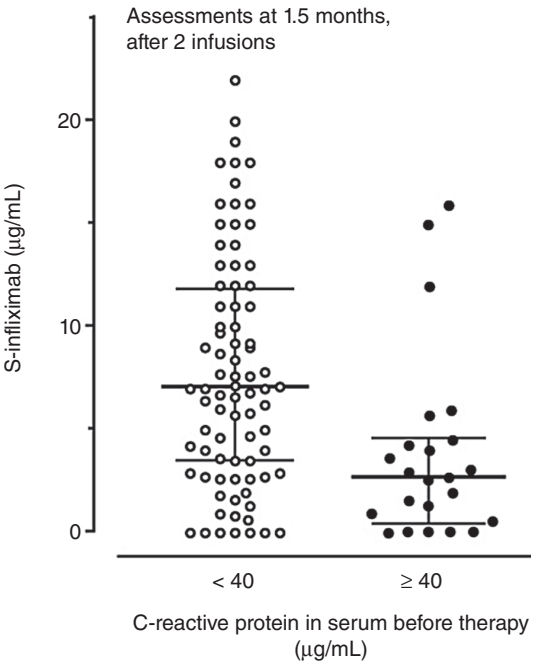

Fig. 5.4 Interindividual variations in infliximab trough levels and effect of pretreatment disease activity. Each dot represents individuals in a cohort of RA patients treated with two infusions of 3 µg/kg infliximab [13]. TNF-binding capacities of patient sera were assessed by RIA and shown as infliximab levels affording equivalent TNF-binding. Median values and quartiles are shown. Trough infliximab levels differed significantly between patients with low and high disease activity assessed by systemic levels of C-reactive protein before therapy ($P = 0.0004$; Mann–Whitney rank sum test).

patients (11%) maintaining clinical effect had values below this level ($P = 0.0003$; Fisher's exact test).

In contrast to infliximab, which is administered by intravenous infusion, adalimumab and golimumab are administered subcutaneously. In these cases, the bioavailability issues are likely to be even more problematic and worsened with the appearance of ADAs causing local immune complex formation.

5.3.2. Assays for ADAs to TNF Inhibitors

Both product- and host-related factors have documented impact on the human immune responses to protein therapeutics [27–31]. The use of anti-TNF antibody constructs for prolonged therapies of immunoinflammatory diseases is no exception. Indeed, ADA responses have been described much more frequently in recent years, and it is likely that the extent of the problem still is not fully realized [11, 32].

It is readily understandable that ADAs may be induced in patients receiving repeated injections of therapeutic proteins containing amino acid sequences originating from other species, as in the case of mouse or chimeric mouse–human anti-TNF drugs. Such ADAs are sometimes called human anti-mouse antibodies (HAMA) or human anti-chimeric antibodies (HACA). However, humanized and even "fully human" antibodies may also be immunogenic, giving rise to human anti-human antibodies (HAHA). Except in a few cases where naturally occurring (auto)antibodies to TNF can be detected in apparently healthy individuals [33], anti-TNF antibodies are not normally part of our antibody repertoire. Anti-idiotypic ADAs may therefore be induced by all human antibody constructs that bind to TNF through the variable parts of their heavy and light chains. In addition, all antibody constructs consisting of human IgG-Fc and/or kappa light chains may provoke antiallotypic antibody responses [10]. IgG allotypes are minor differences in the primary amino acid sequence between molecules of an IgG subclass that occur throughout a species but with different frequencies in different ethnic groups [34]. These allotypic determinants (allotopes) are inherited in a Mendelian pattern, and patients receiving antibodies with "foreign" allotopes may therefore respond with production of antiallotypic antibodies [10, 16, 18].

ELISA is also the most frequently used format for detection of ADAs. Standard ELISAs for detection of antibodies are generally sensitive, accurate, and reproducible when used to detect antibodies *in vitro*, where interference by serum factors is absent or at least limited and often easy to control.

In one setup, Fab′ or F(ab′)2 fragments of the anti-TNF antibody are coupled to plastic, and developed with a tagged mAb against human Fc-γ (Fig. 5.5). Depending on the detection antibody, this assay may reveal ADAs of various isotypes (IgG, IgM, and IgA). Unfortunately, this setup has disadvantages in that it will not report the presence of ADAs binding to the Fc portion of an anti-TNF antibody, for example, antiallotypic ADAs. Furthermore, sera even from healthy individuals contain antibodies that react

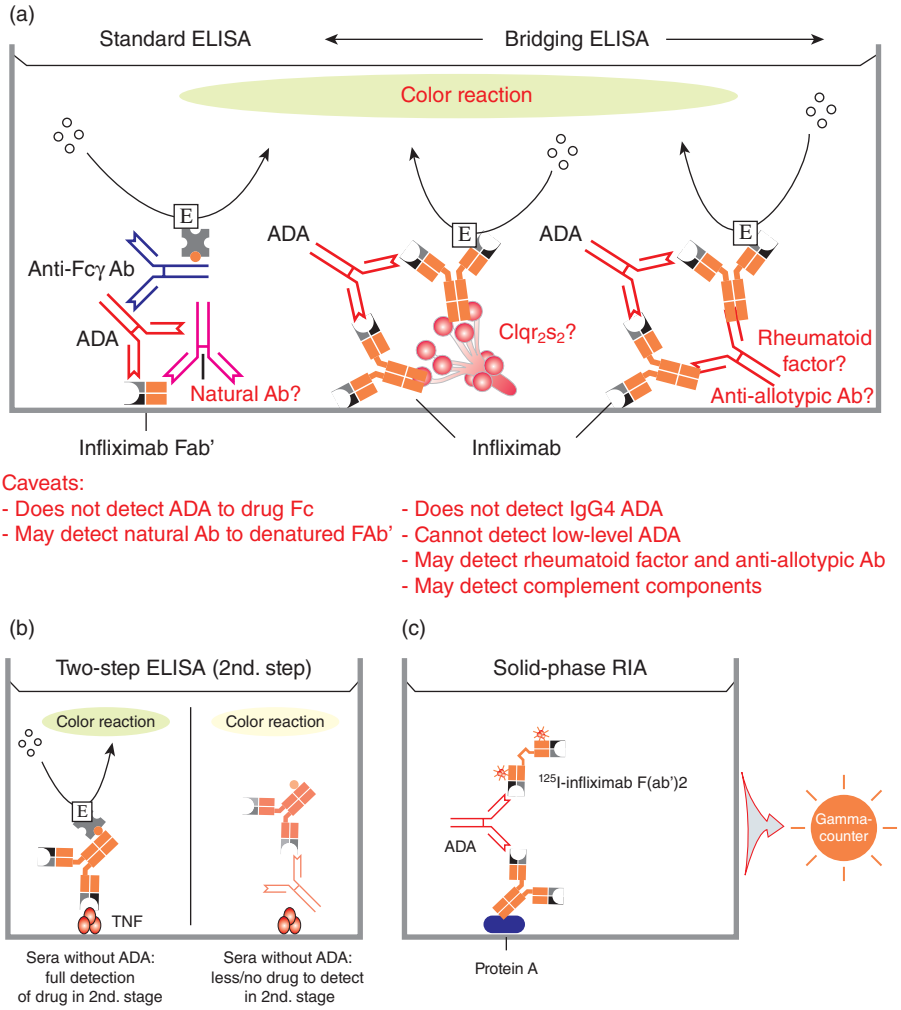

Fig. 5.5 Various techniques for the measurement of ADAs to TNF inhibitors. Examples and caveats related to detection of ADAs by solid-phase ELISA; in this case ADAs directed against infliximab (A), two-step ELISA for ADAs against infliximab (B), and solid-phase bridging radioimmunoassay (RIA) for ADAs against infliximab (C). In the two-step ELISA, ADAs and biotinylated infliximab are precipitated in the first step; unbound infliximab is then measured in the illustrated second stage. (See color insert.)

with solid-phase-immobilized Fab′ and F(ab′)2 fragments, yielding false-positive results for ADA [35, 36].

Anti-drug antibodies have also been detected by a two-stage technique, where ADAs in serum remove biotinylated drug during a first-stage incubation, reducing the amount of free (tagged) drug that may be captured to solid-phase human TNF in the second stage [37] (Fig. 5.5). While it is an advantage that the binding between ADA and drug occurs in solution, the technology can only be used in the absence of detectable quantities of drug.

ELISAs are often problematic when assessing anti-TNF drugs and ADAs in biological fluids, and it is an added difficulty that the antigen itself is an antibody. Recognition of these problems has led to the development of bridging-ELISAs for the measurement of ADAs. This setup utilizes the multivalency of most antibodies so that drug bound to a solid phase can be "bridged" by ADAs to biotinylated or enzyme-tagged drug in the detection phase. Bridging ELISAs have gained in popularity mainly because of their simplicity. However, false-positive results may be encountered due to cross-binding of IgG by complement components and/or rheumatoid factors (Fig. 5.5). It is also a drawback for clinical use that bridging ELISAs cannot detect functionally monovalent antibodies such as IgG4 [10, 38]. This is particularly relevant during long-term "immunizations." For example, high-level IgG4 ADAs are frequently encountered in patients receiving prolonged anti-TNF therapies [16]. The clinical significance of this is that monovalency does not prevent IgG4 ADAs from neutralizing TNF-binding or interfering with drug absorption from injection sites, pharmacokinetics, or delivery to sites of inflammation.

Bridging ELISAs generally benefit from low background levels, which tend to increase sensitivity. Unfortunately, this is to a certain extent counteracted by the relatively high density of antigen that is coated to the solid phase of the assay. As shown in Figure 5.6, ADAs at low levels will not be able to bridge the drug, resulting in false-negative read-outs.

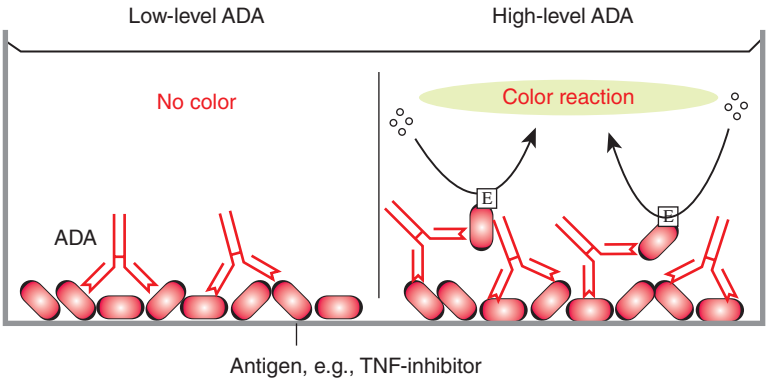

Fig. 5.6 Inability of bridging ELISAs to assess low-level ADAs. (See color insert.)

Other investigators have utilized an RIA where solid-phase-immobilized protein A is used to capture the anti-TNF antibody and then measure radioactivity after addition of radiolabeled Fab′ or F(ab′)2 fragments of the drug [17] (Fig. 5.5C). While it is an advantage that the drug is not denatured by direct solid-phase absorption, this approach cannot detect IgG4 ADAs or ADA binding to the Fc part of the anti-TNF antibody. It is also worth noticing that protein A preferentially binds to Fc-γ1 and Fc-γ2 so that this method only allows detection of ADAs against drugs with intact Fc parts and constructed from the human IgG1 and IgG2 subclasses.

To alleviate some of the problems mentioned here, we have developed an RIA and an enzyme-immunoassay (EIA) for quantification of TNF inhibitors and ADAs in serum and plasma samples [10, 13, 16]. These assays have been developed as fluid-phase assays in an attempt to reflect more closely the clinical situation, including binding *in vivo* between drugs and TNF, and drugs and ADAs, respectively. It should be noted that these assays are applicable to therapeutic antibodies other than anti-TNF antibodies.

5.3.2.1. Fluid-Phase RIA for ADAs. This RIA was originally developed to monitor immunogenicity of TNF inhibitors, primarily infliximab and adalimumab, and chiefly in patients with RA and Crohn's disease [13, 16, 25, 26]. The test principle is shown in Figure 5.7. It utilizes the fact that currently used anti-TNF antibodies consist of kappa light chains only or, in the case of etanercept, no light chains (Fig. 5.1). This means that any class of ADA bound to radiolabeled drug may be separated from free drug with an antibody to human lambda light chains (ADA bound to etanercept may be separated by antibodies to both lambda and kappa chains). The methodology is believed to yield unbiased data because the ratio between kappa and lambda light-chain antibodies is constant in a polyclonal antibody response [16, 39].

Different ways of separating free from ADA-bound drug were assessed in sera of RA patients treated with infliximab, including molecular size chromatography, immunoprecipitation, and cross-binding of soluble infliximab to solid-phase absorbed infliximab in the presence of ADAs [16]. When testing a number of ADA-positive sera by molecular size chromatography, depletion of IgG reduced the binding of radiolabeled infliximab by more than 80%, showing that the major ADA isotype is IgG. Indeed, molecular size chromatography proved to be the most sensitive assay for ADAs. Biochemical and immunochemical characteristics of ADAs from RA patients treated with infliximab are shown in Table 5.1.

The data obtained when testing a large cohort of RA patients treated with infliximab are illustrative (Fig. 5.8). Eleven of 85 RA patients (13%) tested positive for anti-infliximab ADA after only two injections of drug, that is, as early as 1.5 months after start of therapy. In contrast, 28 of 93 (30%) tested positive after 3 months, and 33 of 75 (44%) after 6 months. As expected, low infliximab levels at this early time point predicted ADA responses and later therapeutic failure and infusion reactions [13].

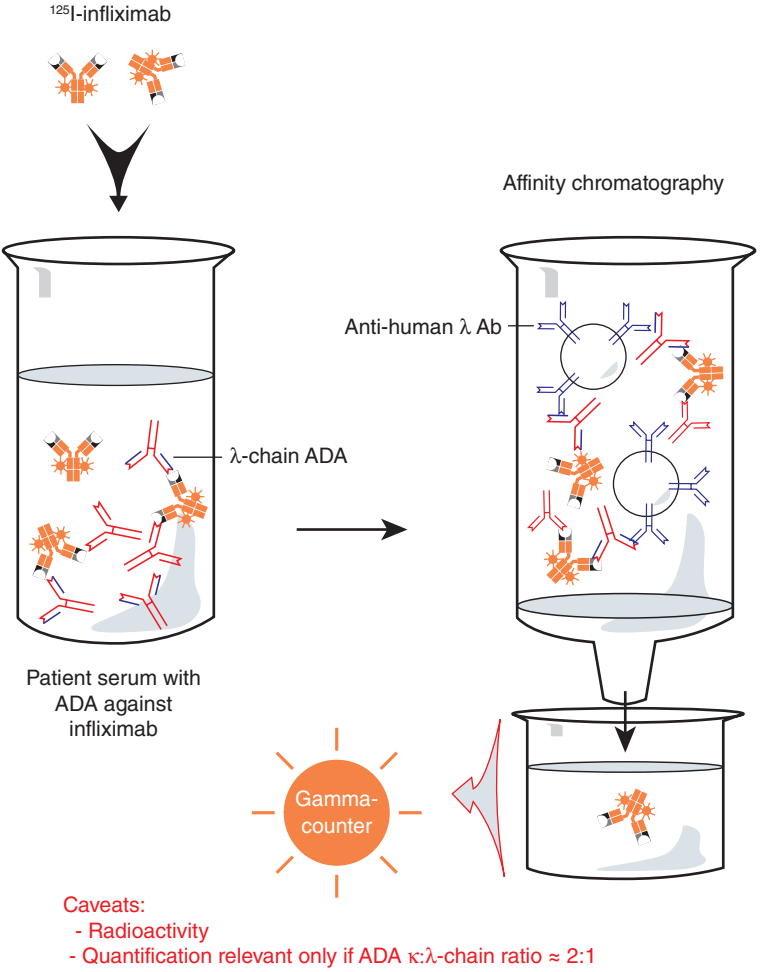

Fig. 5.7 Fluid-phase RIA for ADAs to TNF inhibitors. The example shows RIA for detection of ADAs (all isotypes) from patients treated with infliximab [13, 16]. Patient serum containing ADAs is first incubated with purified radiolabeled infliximab. Free and immunoglobulin-bound tracers are then separated by affinity chromatography using matrix-bound anti-human lambda light-chain antibody. (See color insert.)

Early development of ADAs against infliximab has also been observed in patients with Crohn's disease [21, 26, 42, 43]. Patients with clearly detectable serum levels of ADAs were found to possess significantly lower infliximab levels, and they exhibited shorter response duration and higher risk of infusion reactions.

Concomitant use of immunosuppressive drugs such as methotrexate (MTX) has been shown to increase infliximab trough levels in the circulation [13, 21, 43, 44]. Many investigators point out that the ADA incidences are lowered

TABLE 5.1 RIA-detected ADAs in sera of RA patients treated with infliximab

Light-chain kappa/lambda ratio	2/1
Ig class (isotypes)	IgG (IgG4 constitutes up to 89% in some patients[a])IgE (in up to 18% of RA patients[b])[c]
ADA molecules bound to infliximab	1–3 IgG (depending on infliximab concentration)
Epitopes	Mainly on Fab′
Reactivity	Binds stronger to intact infliximab than to Fab′
Function	Block binding to TNF (neutralizing)
Selectivity	No cross-binding to etanercept or adalimumab

[a]Patients were treated for up to 13 months with infliximab [16].
[b]Reference 40, and unpublished data.
[c]IgE binding to anti-TNF drugs was detected after immunoadsorption of serum IgE to anti-IgE mAb coupled to paramagnetic beads [41]. Radiolabeled TNF inhibitor, with or without unlabeled drug as competitor, was added to the absorbed IgE, and the amount of labeled TNF inhibitor bound to IgE was then determined. Background binding was always less than 0.5% of total counts per minute (cpm) added.

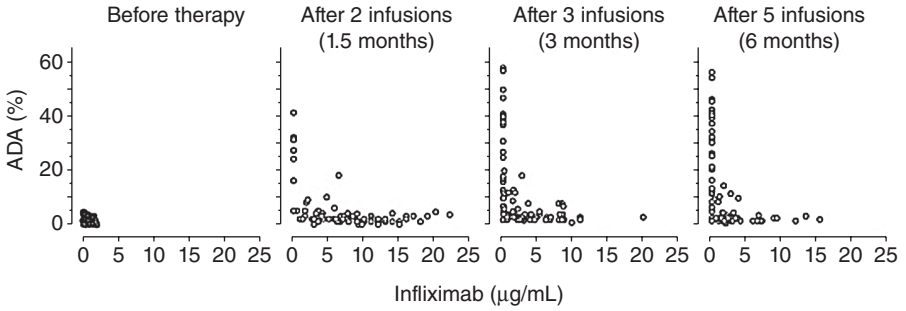

Fig. 5.8 Development of ADAs and association with circulating drug levels in patients treated with infliximab. ADA levels were measured in sera from 106 RA patients treated intravenously with 3 mg infliximab per kilogram bodyweight. ADAs were measured by fluid-phase RIA as percent bound counts per minute (cpm) per total cpm of added radiolabeled infliximab. (Modified from Reference 13.)

significantly by concomitant treatment with MTX and attribute this to the drug's immunosuppressive effect. This, however, is not necessarily correct, as the anti-inflammatory effect of MTX lowers TNF production in inflamed tissues independently of antibody production. Compared with patients not treated with MTX, a reduced "systemic load" of TNF may elevate circulating drug levels and enhance ADA clearance. It is also of note that the incidence of ADA increases with repeated immunizations and that the observed effect of MTX on ADA levels may be transient and therefore of less clear clinical benefit [13].

Our RIA may easily be modified to measure ADAs and cross-reactivity against other anti-TNF antibodies. This is particularly relevant in secondary nonresponders with high ADA levels, and particularly so in patients experiencing serious side effects. Thus, cross-reactivity testing before shifting to another anti-TNF drug could minimize the risk of similar reactions after shifting.

5.3.2.2. Fluid-Phase EIA for ADAs. The principle of this assay is shown in Figure 5.9. The test format is similar to that of the aforementioned RIA in that it keeps the binding between biotinylated drug and ADA in solution. A later double-antibody stage is only used for identification of the previously formed immune complexes between biotinylated drug and ADAs containing lambda light chains. Experiments using infliximab and ADAs from RA patients treated with infliximab show that the EIA performs similarly to that of the RIA with the same level of enzyme-tagged drug. The advantage of this assay is of course that it obviates the use of radioactive isotopes while mimicking antigen–antibody binding processes *in vivo*.

5.4. CLINICAL RELEVANCE OF ADAS TO TNF INHIBITORS

Some investigators believe that the appearance of ADAs is of limited importance, because there are not always observable clinical consequences of ADA development [45]. It is now clear that this view has arisen from clinical experience where several factors have contributed to erroneous interpretations. The use of insufficiently accurate and/or insufficiently sensitive tests contributes to the problem [11, 15, 46]. It is also important to note that the full impact of drug immunogenicity is not realized unless patients are routinely monitored for ADAs or, at the very least, every time side effects or treatment failure occur. If not, clinicians will never know that ADAs could be the underlying cause of therapeutic problems in such patients. Conversely, physicians might not want to monitor patients in clinical remission because they appear to benefit from continued anti-TNF therapy. ADAs may very well develop before and during remissions, and if they are not detected, both patients and physicians may believe that the drug is effective despite its being neutralized by ADAs [10]. Measuring ADAs in these situations would be rational and enable a more economical therapy by minimizing side effects and preventing unnecessary immunization of patients.

The common practice of comparing immunogenicities of drugs targeting the same molecule(s) by the "incidence" of ADA-positivity is also a source of confusion. If unaccompanied by careful information on test formats, for example, cutoffs for ADA-positivity, and if the influence on ADA detection of patient-related issues are not investigated and reported, such data may lead to gross misinterpretations. An illustrative example is the failure to report the time points at which ADA measurements take place. As the frequency of ADA-positives in a patient cohort increases markedly with the length of

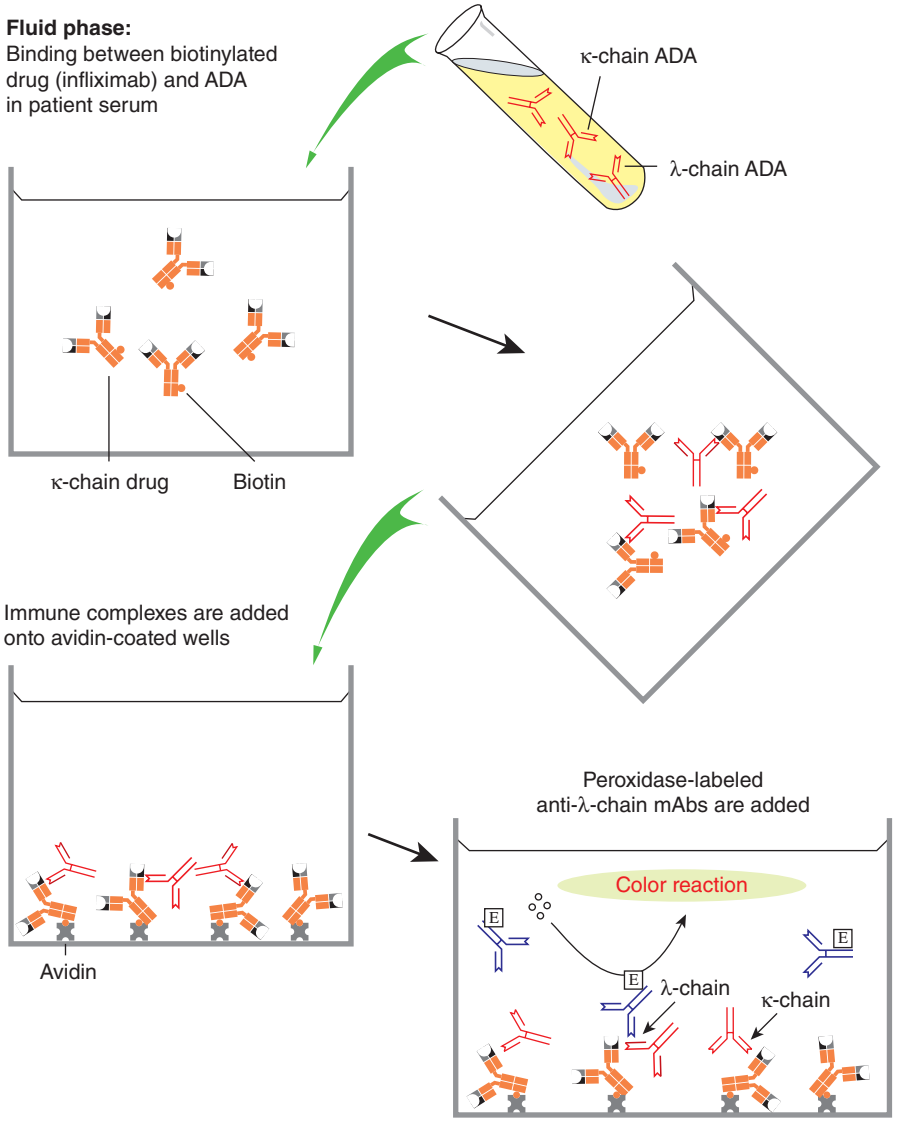

Fig. 5.9 Fluid-phase EIA for detection of ADAs (all isotypes) to TNF inhibitors, from patients treated with infliximab. Patient serum containing ADAs is incubated in solution with biotin-tagged infliximab. Unbound drug, as well as drug in complex with ADA, is then absorbed onto avidin-coated plates; the lambda-containing ADAs are then developed with enzyme-labeled anti-human lambda light-chain antibody. (See color insert.)

therapy (Fig. 5.8), the immunogenicity potential of two drugs cannot be reasonably compared if patients treated with drug A are tested for ADA incidence after 3 months of "immunization" whereas patients receiving drug B are tested after 6 months or later.

Some investigators also believe that only ADAs with a neutralizing effect are of clinical significance. This is not necessarily correct, because *in vitro* non-neutralizing ADAs may "neutralize" patient responses to therapy, as they may adversely affect bioavailability and pharmacokinetics of the drugs [10, 47]. In the case of TNF inhibitors, immune complex formation at injection sites might impair absorption of drugs administered by subcutaneous or intramuscular routes, whether or not ADAs prevent the drug from binding to TNF. This is evidenced by the occurrence of Arthus reactions where ADAs react with residual drug at previous injection sites. Even though these reactions are relatively rare in patients treated with TNF inhibitors, they do occur, and they underscore not only the immunogenicity issue but also the slow and incomplete absorption of an antibody construct administered subcutaneously (see Reference 10). Finally, non-neutralizing ADAs may accelerate systemic removal of TNF inhibitors by formation of immune complexes that are rapidly cleared from the circulation, and ADAs may also prevent TNF inhibitors from reaching inflamed tissues whether or not they interfere with TNF-binding.

It is of interest that ADAs invoked during infliximab therapy of RA patients generally fail to react with adalimumab, another anti-TNF IgG1 construct [16]. Although this does not rule out that cross-reacting ADAs may appear after prolonged infliximab therapy, or that ADAs invoked by other anti-TNF antibodies may interfere with different TNF inhibitors, it may explain why a shift to other anti-TNF drugs is often effective in patients with therapeutic failure or drug intolerance [48–51].

In this chapter, we have only commented upon some of the many factors that may influence the clinical usefulness of assays for antibody-based therapeutics and ADAs. All current technologies pose problems that challenge both therapists and industry and, consequently, health care in general. It should be noted that theoretical considerations and laboratory investigations cannot substitute for proper clinical validation. The number one concern should always be how the various assays perform in the clinical setting, preferably in comparative studies and with special emphasis on how the generated data relate to therapeutic outcome and side effects.

REFERENCES

1. Maini, S.R. (2004). Infliximab treatment of rheumatoid arthritis. *Rheum Dis Clinics North Am*, *30*, 329–347.
2. Vilcek, J., Feldmann, M. (2004). Historical review: cytokines as therapeutics and targets of therapeutics. *Trends Pharmacol Sci*, *25*, 201–209.

3. Moreland, L.W. (2004). Drugs that block tumour necrosis factor: experience in patients with rheumatoid arthritis. *Pharmacoeconomics*, *22*, 39–53.
4. Haraoui, B. (2005). Differentiating the efficacy of tumor necrosis factor inhibitors. *J Rheumatol*, *74* (Suppl.), 3–7.
5. Smolen, J.S., Redlich, K., Zwerina, J., Aletaha, D., Steiner, G., Schett, G. (2005). Pro-inflammatory cytokines in rheumatoid arthritis: pathogenetic and therapeutic aspects. *Clin Rev Allergy Immunol*, *28*, 239–248.
6. Sandborn, W.J. (2006). What's new: innovative concepts in inflammatory bowel disease. *Colorectal Dis*, *8* (Suppl. 1), 3–9.
7. Scott, D.L., Kingsley, G.H. (2006). Tumor necrosis factor inhibitors for rheumatoid arthritis. *New Engl J Med*, *355*, 704–712.
8. Bendtzen, K., Ainsworth, M., Steenholdt, C., Thomsen, O.O., Brynskov, J. (2009). Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. *Scand J Gastroenterol*, *44*, 774–781.
9. Strand, V., Kimberly, R., Isaacs, J.D. (2007). Biologic therapies in rheumatology: lessons learned, future directions. *Nature Rev Drug Discov*, *6*, 75–92.
10. Bendtzen, K. (2008). In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Møller, E.H, eds. Springer, New York, pp. 189–203.
11. Shankar, G., Shores, E., Wagner, C., Mire-Sluis, A. (2006). Scientific and regulatory considerations on the immunogenicity of biologics. *Trends Biotechnol*, *24*, 274–280.
12. European Medicines Agency (EMEA). (2008). Biologics Working Party report to the CHMP. Beta-interferons and neutralising antibodies (in multiple sclerosis), 1–3. Available at http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/11/WC500015395.pdf
13. Bendtzen, K., Geborek, P., Svenson, M., Larsson, L., Kapetanovic, M.C., Saxne, T. (2006). Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. *Arthritis Rheum*, *54*, 3782–3789.
14. Ternant, D., Aubourg, A., Magdelaine-Beuzelin, C., Degenne, D., Watier, H., Picon, L., Paintaud, G. (2008). Infliximab pharmacokinetics in inflammatory bowel disease patients. *Ther Drug Monit*, *30*, 523–529.
15. Bendtzen, K. (2003). Anti-IFN BAb and NAb antibodies: A minireview. *Neurology*, *61* (Suppl. 5), S6-S10.
16. Svenson, M., Geborek, P., Saxne, T., Bendtzen, K. (2007). Monitoring patients treated with anti-TNF-alpha biopharmaceuticals—assessing serum infliximab and anti-infliximab antibodies. *Rheumatology*, *46*, 1828–1834.
17. Aarden, L., Ruuls, S.R., Wolbink, G. (2008). Immunogenicity of anti-tumor necrosis factor antibodies—toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20*, 431–435.
18. Jefferis, R., Lefranc, M.-P. (2009). Human immunoglobulin allotypes. *mAbs*, *1*, 1–7.
19. Tomillero, A., Moral, M.A. (2008). Gateways to clinical trials. December 2008. *Methods Findings Exp Clin Pharmacol*, *30*, 761–782.

20. Zhou, H., Jang, H., Fleischmann, R.M., Bouman-Thio, E., Xu, Z., Marini, J.C., Pendley, C., Jiao, Q., Shankar, G., Marciniak, S.J., Cohen, S.B., Rahman, M.U., Baker, D., Mascelli, M.A., Davis, H.M., Everitt, D.E. (2007). Pharmacokinetics and safety of golimumab, a fully human anti-TNF-alpha monoclonal antibody, in subjects with rheumatoid arthritis. *J Clin Pharmacol*, *47*, 383–396.
21. Baert, F., Noman, M., Vermeire, S., Van Assche, G., D'Haens, G., Carbonez, A., Rutgeerts, P. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *New Engl J Med*, *348*, 601–608.
22. Wolbink, G.J., Voskuyl, A.E., Lems, W.F., de Groot, E., Nurmohamed, M.T., Tak, P.P., Dijkmans, B.A., Aarden, L. (2005). Relationship between serum trough infliximab levels, pretreatment C reactive protein levels, and clinical response to infliximab treatment in patients with rheumatoid arthritis. *Ann Rheum Dis*, *64*, 704–707.
23. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Stapel, S., Lems, W.F., Aarden, L., Dijkmans, B.A., Tak, P., Wolbink, G.J. (2007). Clinical response to adalimumab: the relationship with anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis*, *66*, 921–926.
24. West, R.L., Zelinkova, Z., Wolbink, G.J., Kuipers, E.J., Stokkers, P.C., Van der Woude, C.J. (2008). Immunogenicity negatively influences the outcome of adalimumab treatment in Crohn's disease. *Alimentary Pharmacol Ther*, *28*, 1122–1126.
25. Radstake, T.R.D.J., Svenson, M., Eijsbouts, A.M., van den Hoogen, F.H.J., Enevold, C., van Riel, P.L.C.M., Bendtzen, K. (2009). Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. *Ann Rheum Dis*, *68*, 1739–1745.
26. Ainsworth, M.A., Bendtzen, K., Brynskov, J. (2008). Tumor necrosis factor-alpha binding capacity and anti-infliximab antibodies measured by fluid-phase radioimmunoassays as predictors of clinical efficacy of infliximab in Crohn's disease. *Am J Gastroenterol*, *103*, 944–948.
27. Han, P.D., Cohen, R.D. (2004). Managing immunogenic responses to infliximab: treatment implications for patients with Crohn's disease. *Drugs*, *64*, 1767–1777.
28. Schellekens, H., Casadevall, N. (2004). Immunogenicity of recombinant human proteins: causes and consequences. *J Neurol*, *251* (Suppl. 2), II4–II9.
29. Tangri, S., Mothe, B.R., Eisenbraun, J., Sidney, J., Southwood, S., Briggs, K., Zinckgraf, J., Bilsel, P., Newman, M., Chesnut, R., Licalsi, C., Sette, A. (2005). Rationally engineered therapeutic proteins with reduced immunogenicity. *J Immunol*, *174*, 3187–3196.
30. Cheifetz, A., Mayer, L. (2005). Monoclonal antibodies, immunogenicity, and associated infusion reactions. *Mount Sinai J Med*, *72*, 250–256.
31. Teillaud, J.L. (2005). Engineering of monoclonal antibodies and antibody-based fusion proteins: successes and challenges. *Expert Opin Biol Ther*, *5* (Suppl. 1), S15–27.
32. Watier, H. (2005). Variability factors in the clinical response to recombinant antibodies and IgG Fc-containing fusion proteins. *Expert Opin Biol Ther*, *5* (Suppl. 1), S29–36.
33. Fomsgaard, A., Svenson, M., Bendtzen, K. (1989). Auto-antibodies to tumour necrosis factor α in healthy humans and patients with inflammatory diseases and Gram-negative bacterial infections. *Scand J Immunol*, *30*, 219–223.

34. Grubb, R., Matsumoto, H., Sattar, M.A. (1988). Incidence of anti-human Ig with restricted specificity in Japanese, Kuwaiti, and Swedish patients with rheumatoid arthritis. *Arthritis Rheum*, *31*, 60–62.

35. Persselin, J.E., Stevens, R.H. (1985). Anti-Fab antibodies in humans. Predominance of minor immunoglobulin G subclasses in rheumatoid arthritis. *J Clin Investig*, *76*, 723–730.

36. Osterland, C.K., Harboe, M., Kunkel, H.G. (1993). Anti-gammaglobulin factors in human sera revealed by enzymatic splitting of anti-Rh antibodies. *Vox Sang* 1963;*8*:133–52. *Vox Sanguis*, *64*, 246–247.

37. Candon, S., Mosca, A., Ruemmele, F., Goulet, O., Chatenoud, L., Cezard, J.P. (2006). Clinical and biological consequences of immunization to infliximab in pediatric Crohn's disease. *Clin Immunol*, *118*, 11–19.

38. van der Neut Kolfschoten, M., Schuurman, J., Losen, M., Bleeker, W.K., Martinez-Martinez, P., Vermeulen, E., den Bleker, T.H., Wiegman, L., Vink, T., Aarden, L.A., De Baets, M.H., van de Winkel, J.G., Aalberse, R.C., Parren, P.W. (2007). Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science*, *317*, 1554–1557.

39. Lam, C.W., Chui, S.H., Leung, N.W., Li, E.K., Lai, K.N. (1991). Light chain ratios of serum immunoglobulins in disease. *Clin Biochem*, *24*, 283–287.

40. Benucci, M., Manfredi, M., Saviola, G., Baiardi, P., Campi, P. (2009). Correlation between atopy and hypersensitivity reactions during therapy with three different TNF-alpha blocking agents in rheumatoid arthritis. *Clin Exp Rheumatol*, *27*, 333–336.

41. Ejrnaes, A.M., Bodtger, U., Larsen, J.N., Svenson, M. (2004). The blocking activity of birch pollen–specific immunotherapy-induced IgG4 is not qualitatively superior to that of other IgG subclasses. *Mol Immunol*, *41*, 471–478.

42. Hanauer, S.B., Wagner, C.L., Bala, M., Mayer, L., Travers, S., Diamond, R.H., Olson, A., Bao, W., Rutgeerts, P. (2004). Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. *Clin Gastroenterol Hepatol*, *2*, 542–553.

43. Vermeire, S., Noman, M., Van Assche, G., Baert, F., D'Haens, G., Rutgeerts, P. (2007). Effectiveness of concomitant immunosuppressive therapy in suppressing the formation of antibodies to infliximab in Crohn's disease. *Gut*, *56*, 1226–1231.

44. Van Assche, G., Magdelaine-Beuzelin, C., D'Haens, G., Baert, F., Noman, M., Vermeire, S., Ternant, D., Watier, H., Paintaud, G., Rutgeerts, P. (2008). Withdrawal of immunosuppression in Crohn's disease treated with scheduled infliximab maintenance: a randomized trial. *Gastroenterology*, *134*, 1861–1868.

45. Cassinotti, A., Travis, S. (2009). Incidence and clinical significance of immunogenicity to infliximab in Crohn's disease: a critical systematic review. *Inflamm Bowel Dis*, *15*, 1264–1275.

46. Hennig, C., Rink, L., Fagin, U., Jabs, W.J., Kirchner, H. (2000). The influence of naturally occurring heterophilic anti-immunoglobulin antibodies on direct measurement of serum proteins using sandwich ELISAs. *J Immunol Methods*, *235*, 71–80.

47. Lobo, E.D., Hansen, R.J., Balthasar, J.P. (2004). Antibody pharmacokinetics and pharmacodynamics. *J Pharmacol Sci*, *93*, 2645–2668.

48. Hansen, K.E., Hildebrand, J.P., Genovese, M.C., Cush, J.J., Patel, S., Cooley, D.A., Cohen, S.B., Gangnon, R.E., Schiff, M.H. (2004). The efficacy of switching from etanercept to infliximab in patients with rheumatoid arthritis. *J Rheumatol*, *31*, 1098–1102.

49. Solau-Gervais, E., Laxenaire, N., Cortet, B., Dubucquoi, S., Duquesnoy, B., Flipo, R.M. (2006). Lack of efficacy of a third tumour necrosis factor alpha antagonist after failure of a soluble receptor and a monoclonal antibody. *Rheumatology (Oxford)*, *45*, 1121–1124.

50. Haraoui, B., Keystone, E.C., Thorne, J.C., Pope, J.E., Chen, I., Asare, C.G., Leff, J.A. (2004). Clinical outcomes of patients with rheumatoid arthritis after switching from infliximab to etanercept. *J Rheumatol*, *31*, 2356–2359.

51. Sandborn, W.J., Rutgeerts, P., Enns, R., Hanauer, S.B., Colombel, J.F., Panaccione, R., D'Haens, G., Li, J., Rosenfeld, M.R., Kent, J.D., Pollack, P.F. (2007). Adalimumab induction therapy for Crohn disease previously treated with infliximab: a randomized trial. *Ann Intern Med*, *146*, 829–838.

6

CONFIRMATORY IMMUNOGENICITY ASSAYS

Eric Wakshull and Daniel Coleman

6.1. INTRODUCTION

The assessment of an immune response to a therapeutic protein is considered a part of the routine safety evaluation for both nonclinical and clinical drug development by worldwide regulatory agencies as well as industry. In fact, consideration of the immunogenicity of a protein therapeutic is considered in virtually all phases of the drug discovery and development process. The level of interest and concern about immunogenicity can be measured by the numerous national (US) and international (non-US) conferences dedicated to this topic that occur several times per year and their associated monographs (e.g., [1, 2]), by the increasing number of white papers and guidances that have been or will be issued [3–6], by regulatory guidances [7, 8], and by publications from regulators [9, 10]. This interest and concern is based upon the observed and potential clinical impact of immunogenicity on patient health (e.g., [11–13]) and on therapeutic outcome (e.g., [14–16]). The advent of biosimilar products has generated significant discussion about regulatory pathways for approval. As a result, clinical immunogenicity evaluation of biosimilars in the context of a comparability assessment has become an important topic [17–19]. The spectrum of immunogenicity-associated safety events spans the gamut from benign to catastrophic, with the majority of immunogenic responses being either benign or manageable. It is the link between the presence of antibodies

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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against a protein therapeutic and the safety or efficacy endpoints that provides useful information regarding the clinical impact of such antibodies.

The immunogenicity assessment for a protein therapeutic should be envisaged as a strategy or program, rather than simply one or a set of assays, consisting of the following elements: (1) risk- based assessment; (2) bioanalytical methodology and assay requirements; (3) samples (serum, plasma, etc.); (4) secondary characterization assays (titration, isotyping, epitope mapping, neutralizing activity, etc.); (5) sampling time points; (6) timing of sample analysis (real time or batched); (7) impact assessment or statistical analysis plan (association to relevant clinical endpoints such as pharmacokinetics, pharmacodynamics, safety, efficacy). Together these elements allow for an appropriate immunogenicity assessment and analysis of the data obtained, and importantly a meaningful interpretation of the data in terms of clinical impact. This idea is reiterated from the previous paragraph to emphasize the point that immunogenicity data is relatively uninformative by itself, but can provide useful meaning by its association with relevant clinical (or nonclinical) observations and data.

The focus of this chapter is on confirmatory immunogenicity assays. A discussion of the factors that influence the immunogenicity of a particular therapeutic protein are numerous, complex, and beyond the scope of this chapter. Many articles have been published on this topic (e.g., [20–22]), and the reader is referred to these and references therein for detailed information. Regulatory perspectives on immunogenicity are provided in various chapters in this volume as well as in the white papers and the regulatory guidances and publications previously noted.

6.2. IMMUNOGENICITY ASSAY STRATEGY AND PURPOSE

A typical tiered strategy for assessing immunogenicity is schematized in Figure 6.1 that, in its basic form, has been adopted by most of the biopharmaceutical industry. This schema represents in essence a triage approach to immunogenicity testing, and from top to bottom each assay provides more detailed information about the nature and characteristics of the immune response.

The purpose of the screening assay is to detect all antibodies against the protein therapeutic, regardless of their molecular characteristics or functional impact (see the subsequent sections). The sensitivity of the assay for detecting anti-protein therapeutic antibodies is defined by the assay cut point [3, 6], the assay decision threshold used to determine the positive or negative status of a sample. The cut point is derived from the variability of the assay signal generated by a panel of serum samples representing the drug-naive disease population of interest. The assay sensitivity is then approximated by the concentration of a surrogate positive control antibody, usually an animal-derived polyclonal or monoclonal antibody, that produces a signal at or beyond the cut point. Designed into this assay cut point is a statistical probability of detecting false

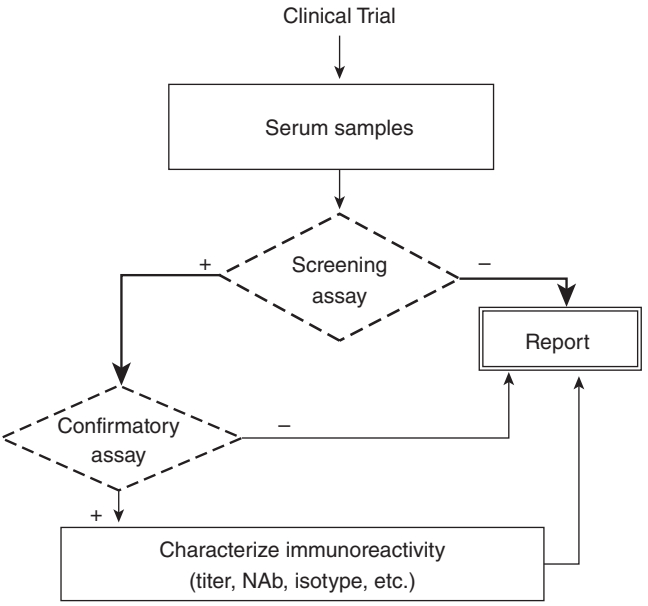

Fig. 6.1 Typical tiered immunogenicity assay strategy. Samples are first tested in a screening assay. Samples with signals above the assay cut-point value are considered positive and are further evaluated in a confirmatory assay, while testing of samples deemed negative in the screening assay is considered completed. Samples with signals that exceed the confirmatory assay cut point are considered positive and are candidates for further characterization, while samples with assay signals that do not exceed the confirmatory assay cut point are reported as negative and not analyzed further.

positive signals at the rate of 5%. This is done to provide evidence that the assay is sufficiently sensitive to detect low levels of antibodies [3]. To further confirm the screening assay positives as "true," as well as to eliminate the false positive samples, a confirmatory assay is used as a second tier in the triage schema. Samples that are confirmed as "true" positives are then considered for further characterization, for example, quasi quantitation of the response via a titration assay or their ability to neutralize the therapeutic protein in an *in vitro* cell-based or ligand-binding functional assay. Neutralizing-antibody assays are the subject of an industry white paper [4] and are discussed in greater detail in other chapters of this volume.

6.3. CURRENT APPROACH TO CONFIRMATORY ASSAY DESIGN

The most commonly used confirmatory assay design utilizes the screening assay format with the addition of spiked excess unlabeled protein therapeutic into the sample prior to being run in the assay. Basic antigen–antibody biochemistry suggests the binding interaction between the protein therapeutic

(antigen) and an antibody against the protein therapeutic should be specific and saturable and thus able to be competed by unlabeled antigen in an assay system. Thus, competitive inhibition of the assay signal by added drug demonstrates the binding to be specific and saturable. The assumption is that exposure-induced binding activity to the protein therapeutic would be due to an immune response against the protein therapeutic, resulting in the production of specific polyclonal antibodies (but there could be other exposure-induced nonantibody interfering substances produced as well, such as soluble target—see Section 6.7, item 4). This approach is often referred to as immunodepletion, but in fact no depletion of a binding component from the sample is actually occurring. Rather, this is a straightforward competitive-binding format.

Another approach, less often utilized due to its greater procedural complexity and increased sample processing steps, is to deplete samples of immunoglobulins (Igs) by affinity chromatography methods (e.g., using protein A/G beads), leading to signal reduction if anti-protein-therapeutic antibodies are present. In this format, the binding antibodies are in fact depleted from the sample, and this process has been termed immunodepletion.

Thus, the competitive-binding confirmatory assay is a variation of the screening assay, but it provides different information about a sample (see Section 6.4). However, confirmatory assays per se are not routinely addressed in the published literature. Most primary articles from clinical trials, nonclinical safety studies, or even methods papers provide minimal experimental details and these usually only on the screening assay. The recent European Medicines Agency guidance [8] mentions confirmatory assays but only goes so far as to say that repeating the screening assay is not confirmatory. The immunonogenicity validation white paper by Shankar et al. [6] briefly describes "specificity confirmatory assays," and this is perhaps more appropriate nomenclature given the experimental design described. The seminal white paper by Mire-Sluis et al. [3] discusses assay specificity in terms of the ability of the analytical method to detect an analyte of interest in the presence of potentially interfering substances, but the focus is on residual drug in a sample as one of many potential interfering substances that screening assay developers need to assess.

As a consequence, the assay developer has license to investigate the use of whatever assay design and/or technology is most suitable for their molecule, their program needs, and their operational constraints. Discussions of various commonly used immunogenicity screening assay designs and their pros and cons have been published ([3, 23]; this volume). From a cost and throughput perspective, the following primary assays are commonly used. (1) The indirect enzyme-linked immunosorbent assay (ELISA) immobilizes antigen (in this case, the protein therapeutic of interest) on a microtiter plate either directly or via a specific capture molecule, followed by sample incubation then detection with an enzyme-labeled anti-human Ig antibody purchased from commercial sources. These assays often use sequential incubation steps and are thus generally less sensitive to low-affinity antibodies (Abs), are sometimes

less tolerant of residual drug in the sample, and may require more specific reagents to detect all isotypes as well as the surrogate positive control, but they have the advantage of identifying the detected molecule as an antibody. (2) In bridging ELISAs, the antigen is labeled typically with either biotin (for the immobilization–capture step) or a detection label (e.g., digoxigenin, abbreviated DIG), both labeled species then being combined in solution with the sample to generate a trimolecular complex (bridge) of biotin–drug/Ab/DIG–drug when anti-protein-therapeutic antibodies are present. This complex is captured then detected via enzyme-labeled anti-DIG antibodies. The bridging assay design is a virtual necessity for humanized monoclonal antibodies since anti-human Ig detection antibodies cannot be used. This design has fewer wash steps, allowing for better detection of low-affinity Abs, and by providing for better control of reagent (i.e., labeled drug) stoichiometry in the reaction mixture, has better residual drug tolerance than the stepwise ELISA. However, this design suffers from the disadvantage of detecting any molecule that can bind drug and oligomeric or multimeric (e.g., soluble bivalent target molecules). It should be noted that the bridging format can be performed in a stepwise incubation format similar to the sandwich ELISA, rather than the homogeneous fashion just described. The former approach will generally be more susceptible to residual drug interference and, with the additional wash steps, be less sensitive to low-affinity antibodies.

Alternative technologies that have been usefully employed include radioimmunoprecipitation assays (RIPA) and surface plasmon resonance (a technology currently dominated by the Biacore platform, but others are also available; this technology is also suitable for humanized monoclonal antibodies). Electrochemiluminescent assays use the basic ELISA designs just described but with a different detection technology (this technology was previously available from Bioveris and is currently dominated by Meso Scale Devices).

6.4. ORTHOGONALITY

We apply the term "orthogonality" to the screening and confirmatory assays not in its mathematical sense of systems at right angles, but in the more general sense of systems being independent, nonredundant, and nonoverlapping (in information content). This meaning also aligns with the concept of statistical orthogonality, discussed later in this section. For the confirmatory assay to be useful and cost effective, the assay must provide information different from the screening assay. At first glance, running the screening assay on samples that have merely had excess drug spiked in would not appear to be "orthogonal"; probably many scientists consider that term to imply the use of a different analytical technology. Instead we think of techniques such as SDS–polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography (SEC), multiangle light scattering (MALS), and analytical

ultracentrifugation (AUC) as examples of orthogonal methodologies, providing different approaches to obtaining molecular size information. However, while these techniques will provide somewhat different information, they will also likely provide nonidentical values for molecular size of a molecule because of how it interacts with the system components (buffer, matrix, detection, etc.) that are inherent to each technology. A confirmatory assay that was orthogonal in this sense might indeed provide qualitatively different information, as different methodologies (e.g., ELISA versus surface plasmon resonance) will have differing ability to detect the complex polyclonal immune response and might provide quite different response outcomes for some samples [24]. Anyone who has compared results from the same sample set run in two different assay methodologies or technologies has probably noted this potential for a confounded outcome.

Screening and confirmatory assays, as described here, in fact do provide different information on the antibody–protein therapeutic interaction being detected and, in the context of the analytical techniques just mentioned, do so with a minimum of changes in system variables: added excess drug in the buffer. Thus, a confirmatory assay that utilizes the screening assay platform obviates the potential for contradictory outcomes just noted. Screening assays measure a signal increase due to the binding of an antibody to the protein therapeutic. Confirmatory assays measure the specific inhibition of binding in the presence of the unlabeled protein therapeutic as a binding "competitor." A sample is considered positive in the screening assay when its signal is above a cut-point value derived from the variability of the naive patient population. A sample is considered positive in the confirmatory assay if its value falls below a cut point derived, as described in Section 6.5, from the variation in the ratio of spiked to unspiked samples from the naive patient population.

From a statistical point of view, the orthogonality of the two methods is demonstrated if the values for negative test samples derived from the methods do not correlate. Such a result is shown in Figure 6.2A, which plots the screening assay score versus a confirmatory assay score from 48 naive disease-state serum samples (see legend for descriptions of the screening and confirmatory scores). The lack of correlation is clear from visual inspection of the scatterplot array of the data and the low correlation coefficient. If the two sets of results from the samples did correlate, the false positives found in the screening assay would be found again in the confirmatory assay and there would be no reduction in the overall false positive rate and no reason to run the confirmatory assay! In contrast, if one plots screening scores of a sample set run in repeated screening assays against each other, a clear linear correlation should be observed (Fig. 6.2B)—this correlation demonstrates reproducibility of the screening assay, which gives us confidence that we can make a correct call, positive or negative, based on analyzing a sample in a single assay run. This is the standard operational approach to sample testing. The failure to demonstrate the types of relationships shown in Figure 6.2A,B should prompt the assay developer to re-evaluate their method.

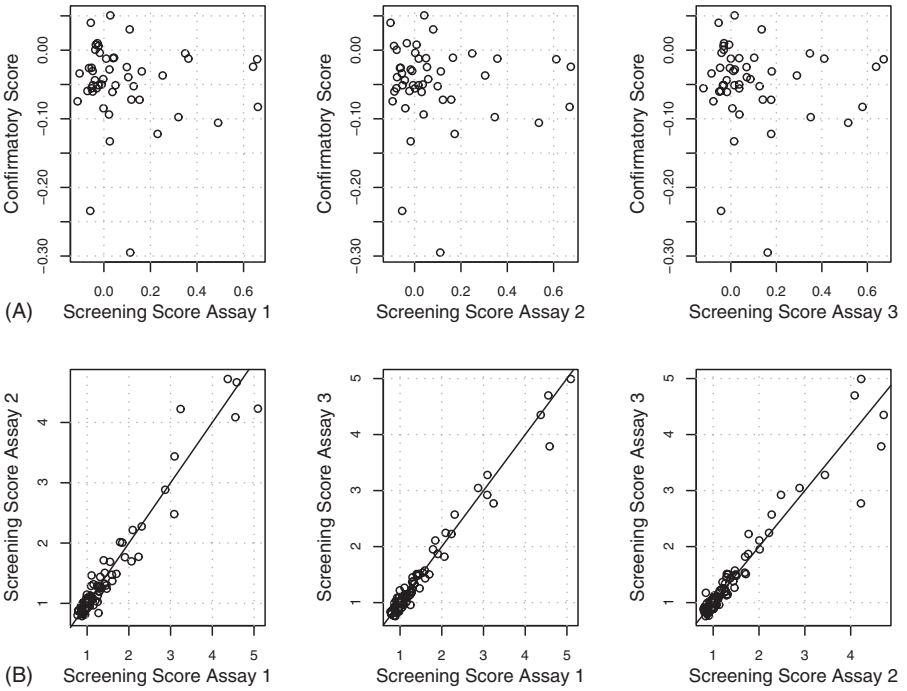

Fig. 6.2 (A) Comparison of confirmatory and screening scores. Confirmatory scores {log(Unspiked Sample Signal) – log(Spiked Sample Signal)} and three independent assay runs of screening scores {log(Sample Signal) – log(Neg. Ctrl. Signal)} from 48 untreated patient samples were plotted against each other. The lack of correlation (Pearson correlation = –0.06, –0.07, and –0.07, respectively) between the scores indicates that the confirmatory and screening assays are orthogonal; that is, they measure different qualities or properties of the samples. Hence false positive samples from the screening assay should be detected as negative samples in the confirmatory assay. (B) Comparison of screening scores. Screening scores on 100 untreated patient samples (includes the 48 samples run in the confirmatory assay) from three independent assay runs were plotted against each other. The high correlation (Pearson correlation = 0.97, 0.99, and 0.96, respectively) between the scores indicates that the screening assay is reproducible.

6.5. SETTING THE CUT POINT

Using the screening assay format simplifies confirmatory assay development, with only the amount of spiked drug that should be added to the sample requiring experimental evaluation. The amount of added drug spiked into a sample needs to be determined empirically, using a surrogate positive control, in order to know whether it can effectively compete for binding with some estimated high level of anti–protein therapeutic antibody that might be present in a real sample. As an example, we have found that a final concentration of

20–100 µg/mL *in-well* concentration of added drug appears sufficient for providing complete inhibition of even high levels (tens of micrograms per milliliter in neat serum) of positive control. However, the reader should be aware of certain caveats in this regard (see Section 6.7, Assay Challenges).

A key feature of this and all other immunogenicity assays is determination of the assay cut point. The cut point is the decision threshold at which an assay signal for a given sample determines whether it is positive or negative, and defines the assay sensitivity as well, as noted earlier. The method for determining the confirmatory assay cut point described in this section has been described elsewhere [6] and makes use of the paradigm established for the screening assay cut point [3]. In essence, the variability of assay response in the sample set from the target population is assessed, and the assay signal at the 95th percentile of this distribution is calculated. Any signal below this value is considered positive. While the screening assay cut point is designed to allow a 5% false positive rate, a lower false positive rate for the confirmatory cut point has been proposed [6]. However, there is some measure of arbitrariness—and assumption of risk—to whatever value is chosen. This approach has the virtues of (a) being consistent with the screening assay approach, (b) providing a conservative cut point depending on the percentile employed, (c) being easily defensible scientifically, (d) being broadly applicable to many methodological approaches, and (e) when the screening and confirmatory assays are orthogonal, providing an overall untreated positive rate (UTPR) that is the product of the individual UTPR of the two assays. There are alternative methods that have been adopted, and we will briefly describe some of them in the next section.

A set of drug-naive serum samples derived from the target disease population, generally purchased from a commercial vendor, is run in the optimized confirmatory assay. There are two important assumptions about these samples that should be noted. First, that they are representative of the intended clinical population; second, that they are true negative samples. If the confirmatory assay uses the screening assay platform, it is recommended that the panel of samples be run concurrently both with and without added excess drug. This accomplishes two objectives: (a) determination of the screening assay cut point; (b) the ability to normalize the spiked sample result with the unspiked sample result for determining the confirmatory assay cut point. The number of individual samples and number of times the sample set is run should be discussed with a qualified statistician. However, we typically use 50–100 individual samples that will be run unspiked 3 separate times, while spiked samples will be run once. The reason for running the spiked samples only once is that the "within-sample" normalization process removes essentially all but well-to-well variability since each sample is normalized (i.e., compared to the unspiked sample) to itself on a single plate during the confirmatory assay.

The confirmatory assay score for any one sample is given by the ratio of the spiked sample to the unspiked sample. This value is calculated for all the samples in the set. Confirmatory assay scores can be log-transformed to better

approximate a normal distribution if the nontransformed data are not normally distributed. This allows for a simple parametric estimate of the percentiles: 95% = (average score) – (1.645 × standard deviation); 99% = (average score) – (2.236 × standard deviation). The average and standard deviation are computed from all the ratio scores of the sample set.

An example of the type of raw data obtained is provided in Figure 6.3A, which plots the assay signal of the unspiked versus the spiked samples. The line represents identity, that is, unspiked = spiked. The data points not on the identity line show the change in signal following spiking, and these generally show a small decrease in assay signal. Note that the distance from the identity line is reasonably similar irrespective of the absolute unspiked values. This is equivalent to the outcome shown in Figure 6.2A, which shows a lack of correlation between the screening score (unspiked) and the confirmatory score (spiked). In other words, unspiked assay *signals* correlate with spiked assay *signals* so that their *ratio* (confirmatory score) is relatively the same regardless of the magnitude of the unspiked signal. By computing this ratio, individual and plate variability from the confirmatory score are effectively removed, leaving primarily well-to-well variability. From this data, a frequency histogram of confirmatory scores log[(spiked score)/(unspiked score)] and a 95% UTPR cut point are drawn (Fig. 6.3B).

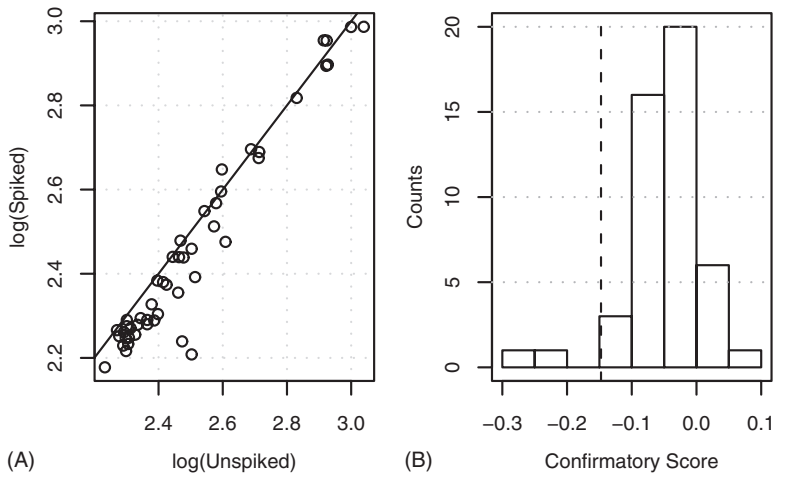

Fig. 6.3 (A) Comparison of unspiked versus spiked samples in a confirmatory assay. Scatter plot of log(Unspiked Sample Signal) versus log(Spiked Sample Signal). The high correlation (correlation = 0.97) shows that the spiked signal retains the same variation due to sample biology as the unspiked samples. (B) Histogram of the confirmatory scores. The vertical dotted line at –0.15 is the cut point; samples with scores less than this cut point are confirmed positive. The cut point was set to control the untreated (or false) positive rate at 5%. It was computed as the average minus 1.645 times the standard deviation of the confirmatory scores on 48 untreated patient samples.

Having established the confirmatory assay cut point, the assay developer is encouraged to evaluate its effectiveness using individual samples spiked with a low amount of positive control antibody. These should confirm as true positives for most samples tested. "True" positives from drug-exposed patients cannot be used to verify cut points since their status as "true" positives is determined by these immunogenicity assays themselves—a circular exercise.

6.6. ALTERNATIVE METHODS FOR SETTING THE ASSAY CUT POINT

As mentioned previously, there are other methods that have been described to determine a cut point for competition-based confirmatory assays.

Some labs will use a sample-specific IC_{50} value as a cut point—a sample is deemed positive if its assay signal is inhibited at or below a level midway between its unspiked signal value and the negative control or screening cut point. While simple and objective, this approach requires that a 50% inhibition be obtained which may not (a) occur for some "true" positive samples or (b) provide the most sensitive cut point if the population variability allows for a cut point with less inhibition.

Other labs prefer to spike individual samples with positive control antibody at a level near the screening assay sensitivity, spike in excess drug, then determine the variability of signal inhibition obtained with an approach similar to the method just described. The reason for this approach is the observation that very low positive samples, with signals near the screening assay cut point, can be difficult to inhibit to the level otherwise determined by simply evaluating signal changes from naive antibody-negative individuals, potentially leading to false negative results. However, this approach has three assumptions of note. First, that the surrogate positive control, usually obtained from a hyperimmunized animal or a mouse monoclonal antibody, used to determine the cut point is representative of the human patient population response in terms of its drug-binding properties. Second, the amount of positive control antibody spiked in to generate the set of individual positives is an appropriate amount for determining the cut point. If more positive control is used, the cut point will be larger and vice-a-versa (e.g., the percentage inhibition of a signal of 500 to a background of 50, versus inhibition of a signal from 100 to a background of 50), hence the amount of positive control selected by the researcher effectively determines the cut point. Third, this method controls the false negative rate instead of the false positive rate as used to set the screening cut point. These assumptions need to be carefully considered and understood when employing this approach.

A third approach based upon using a *t*-test method for calling a sample positive or negative for antitherapeutic antibodies in the confirmatory assay has been proposed by Neyer et al. [25]. Based upon null hypothesis testing, a sample is declared positive if the Student's *t*-test detects a statistically signifi-

cant difference greater than zero between the unspiked and the spiked samples. They recommend testing three unspiked and three spiked samples. In their approach, the size of the observed signal difference between the spiked and the unspiked samples is irrelevant, only its statistical significance is important. Statistical significance is a complex function of the data. If the sample size is large or the variability within conditions is small, the *p*-value will be small and the Student *t*-test will detect a small but perhaps clinically irrelevant difference between the unspiked and the spiked signal values. Setting the confirmatory cut point to control the UTPR takes the opposite point of view, in which the magnitude of the observed difference is the only concern. A sample is declared positive only if the size of the observed difference is greater than what would be expected if the sample had come from an untreated patient. This approach links the confirmatory cut point to the underlying biology while the *t*-test method does not.

In addition, the *t*-test method assumes that if no antitherapeutic antibodies are present in the sample, the unspiked and spiked scores are exactly the same on average. In our experience, this assumption does not hold with electrochemiluminescent formats, where spiked signals are typically somewhat lower than the unspiked signals in drug-naive samples.

6.7. CONFIRMATORY ASSAY CHALLENGES

There are a number of challenges that assay developers or operational groups might encounter. An exhaustive list is not possible here. A few of these will be described briefly.

1. *Spiked (or unspiked) negative samples have assay signals close to instrument noise or system background levels.* Here we briefly digress to discuss an important issue for screening assays. As noted earlier, it is essential from an operational perspective to have confidence that a screening assay provide a reliable (i.e., reproducible) result on sample status (anti–protein therapeutic positive or negative) from the first test, since samples are usually tested one time. Negative samples are not retested whereas positive samples will be tested in additional assays. Thus, when basing a cut point for a screening assay on the variability of the sample population, it is critical that the variability measured is dominated by the *biology* of the sample and not by the system (instrument, plates, operator, environmental factors, etc.) variability. This can be shown convincingly by demonstrating that screening assay scores from repeat tests of naive samples correlate (Fig. 6.2B). The correlation (reproducibility) is due to the assay measuring the sample biology (i.e., nonspecific binding), with only a negligible component of the signal derived from the assay system. While not formally a screening assay retest, unspiked samples in the confirmatory assay do provide some information about the screening score reproducibility. Obviously we would like the unspiked sample value in the confirmatory assay

to reproduce the value obtained for that sample in the original screening assay. Thus, this issue of the assay signal reflecting sample biology and not system noise is also important for confirmatory assays.

Now we consider this challenge as it pertains to the confirmatory assay. When spiked naive samples have assay signals at or near the instrument background, then the numerator of the confirmatory score ratio is roughly constant and the variability of confirmatory scores used to calculate the confirmatory cut point is determined by the unspiked sample variability, rather than the amount of signal change due to spiked drug. When this is the case, the screening and confirmatory assays are measuring the same thing and the orthogonality of the confirmatory assay is thus lost. This situation can occur if the unspiked naive sample background is close to instrument noise, leading to reproducibility concerns for the screening assay (see above). This can also occur if an excessive amount of spiked drug is used. One approach to solve this problem is to increase the serum concentration so that the background is dominated by the sample biology (i.e., nonspecific binding components), rather than instrument (or system) noise. For bridging assay formats that use dual labels as described earlier, increasing the amount of labeled drug will also raise the baseline signal away from instrument–system noise. Alternatively, the amount of competing drug could be lowered so that a more modest level of signal reduction is obtained.

2. *Confirming antitherapeutic protein antibodies of the IgM isotype.* Regulatory agencies prefer assays capable of detecting antitherapeutic protein antibodies of all isotypes and subtypes. Bridging ELISAs are capable of detecting IgMs. However, standard sandwich ELISAs using anti-isotype detection reagents require specific anti-human IgM reagents to do so, making those assays more complex. It may be possible to use anti-($\kappa+\lambda$) light chain antibodies for this purpose. Assuming that the screening assay can detect IgMs, spiked excess drug may not compete effectively for binding with a high avidity (decavalent) IgM. Unfortunately, having access to an IgM surrogate positive control in order to assess whether a response can be competed is unusual. IgMs are generally of low titer and low (intrinsic) affinity. The clinical significance of anti–protein therapeutic antibodies of the IgM isotype is uncertain. Most significant immunogenic responses will likely switch isotype to IgGs if they mature. We know of no operationally efficient and effective approach to this problem.

3. *High titer responses.* It is possible that an immune response is so large that the chosen concentration of spiked drug would not effectively inhibit the assay signal beyond the cut point. A high screening signal that appears following exposure to drug but cannot be confirmed by the established confirmatory assay criteria should be further investigated. There are a number of approaches to dealing with this possibility. First, evaluate secondary endpoints to determine possible impact that might be expected of a strong immunogenic response; for example, altered pharmacokinetics or pharmacodynamics or even adverse

events might indicate a true positive response that can then be evaluated further if desired. Second, samples that give high signals but cannot be competed can be further diluted prior to spiking to see if competiton can be effected.

4. *Soluble drug target interference produces false positives.* This challenge is particularly prevalent for the bridging ELISA, where any bivalent (multivalent) specific binding partner can form a detectable bridge complex. Such complexes can effectively be competed by excess drug and thus confirmed in a confirmatory assay of the design described here. Both soluble targets, as well as cell surface receptors that can be released from cells in a soluble form have the potential to generate such false positives. Assay developers need to be alert to this possibility and determine from the literature and experimentally the potential for this occurrence. Two approaches can be suggested to address this problem. First, the soluble target can be depleted from the sample by affinity chromatography prior to sample testing. While effective, this approach increases operational complexity. Second, addition of a "blocking" monoclonal antibody to the target that competes for binding to the soluble target with the drug itself can eliminate formation of labeled-drug–soluble-target complexes in the assay. This has proven quite effective for several molecules, but it has the potential drawback of the blocking antibody also blocking anti-idiotypic or anti–complementarity-determining region (CDR) antibodies in the sample. Even the use of a blocking antibody with a different CDR sequence than the drug cannot rule out the possibility of also blocking anti-drug CDR antibodies. That being said, tests in our laboratory using positive control polyclonal anti-drug CDR antibodies or even a panel of monoclonal anti-CDR antibodies have not shown detection of these positive controls to be impaired by these blocking antibodies.

6.8. CONCLUSIONS

The assessment of the immunogenic response is one aspect of the safety evaluation of protein therapeutic drugs. The immunogenicity strategy commonly employed in the biopharmaceutical industry entails a triage structure in which samples judged positive in a screening assay are run in a confirmatory assay. Confirmed positive samples may then be further characterized by quasi-quantitative titration assays, neutralizing assays, and so on. Confirmatory assays provide additional information for making a call on the anti–protein therapeutic antibody status of a sample, and should also eliminate false positive samples generated by the screening assay cut point design. The lack of correlation between sample screening and confirmatory assay scores is essential in demonstrating that the assays are orthogonal and thus providing complementary and useful information. Like all immunogenicity assays, determination of the decision threshold, the assay cut point, is critically important. One approach that we prefer was presented in detail, but the assay

developer should use their best scientific judgment whether this or a different approach is best suited to their needs. Some of the more common confirmatory assay challenges were addressed, but every assay can surprise its developer with new and unexpected challenges.

One interesting feature of the confirmatory assay design described here is the presence on one plate of both unspiked and spiked samples. When using the screening assay design for the confirmatory assay, this means that the unspiked samples are essentially run in the screening assay format alongside the confirmatory assay format. Therefore, one could choose to simply run this design during primary sample testing to generate both sets of data, and a call of positive or negative made based upon the score generated from both sets. The down side of this approach is that each assay plate will hold fewer samples. If the expected immunogenicity incidence is low and the sample number high, then this design is less efficient than the sequential approach described in the triage strategy (Fig. 6.1).

REFERENCES

1. Brown, F., Lubiniecki, A., Murano, G. (2002). *Biologics 2000—Comparability of Biotechnology Products*. *Developments in Biologicals*, Vol. 109. Brown F, ed. Karger, Basel.
2. Brown, F., Mire-Sluis, A.R. (2003). *Immunogenicity of Therapeutic Biological Products*. *Developments in Biologicals*, Vol 112. Brown F, ed. Karger, Basel
3. Mire-Sluis, A.R., Chen, Y.C., Devanarayan, V., et al. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
4. Gupta, S., Indelicato, S.R., Jethwa, V., et al. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
5. Koren, E., Smith, H., Shores, E., et al. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
6. Shankar, G., Devanarayan, V., Amaravadi, L., et al. (2009). Recommendations for the validation of immunoassays used for the detection of host antibodies against biotechnology products. *J Pharmaceut Biomed Anal*, *48*, 1267–1281.
7. International Conference on Harmonization (ICH). (1997). Safety guidance S6, preclinical safety evaluation of biotechnology-derived pharmaceuticals.
8. European Medicines Agency (EMEA). (2008). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins (EMEA/CHMP/BMWP/14327/2006, effective April 2008).
9. Rosenberg, A.S., Worobec, A. (2005). A risk-based approach to immunogenicity concerns of therapeutic protein products. Parts 1–3. *BioPharm Int*, Nov, Dec 2004, Jan 2005.

10. Swann, P.G., Tolnay, M., Muthukkumar, S., et al. (2008). Considerations for the development of therapeutic monoclonal antibodies. *Curr Opin Immunol*, *20*, 493–499.
11. Casadevall, N., Nataf, J., Viron, B., et al. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New Engl J Med*, *346*, 469–475.
12. Vandhan-Raj, S. (2000). Clinical experience with recombinant human thrombopoietin in chemotherapy-induced thrombocytopenia. *Semin Hematol*, *37* (Suppl. 4), 28–34.
13. Chung, C.H., Mirakhur, B., Chan, E., et al. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-alpha-1,3-galactose. *New Engl J Med*, *358*, 1109–1117.
14. Baert, F., Noman, M., Vermeire, S., et al. (2003). Influence of immunogenicity on the long-term efficacy of infliximab on Crohn's disease. *New Engl J Med*, *348*, 601–608.
15. Cohen, B.A., Oger, J., Gagnon, A., Giovannoni, G. (2008). The implications of immunogenicity for protein-based multiple sclerosis therapies. *J Neurol Sci*, *275*, 7–17.
16. Calabresi, P.A., Giovannoni, G., Confavreux, C., et al. (2007). The incidence and significance of anti-natalizumab antibodies. *Neurology*, *69*, 1391–1403.
17. Pavlovic, M., Girardin, E., Kapetanovic, L., Trouvin, K.H.J.-H. (2007). Similar biological medicinal products containing recombinant human growth hormone: European regulations. *Hormone Res*, *69*, 14–21.
18. Mellstedt, H., Niederwieser, D., Ludwig, H. (2008). The challenge of biosimilars. *Ann Oncol*, *19*, 411–419.
19. Schellekens, H. (2009). Biosimilar therapeutics—what do we need to consider? *Nephrol Dial Transplant Plus*, *2* (Suppl. 1), i27–i36.
20. Schellekens, H. (2002). Bioequivalence and the immunogenicity of biopharmaceuticals. *Nature Rev Drug Discov*, *1*, 457–462.
21. Schellekens, H. (2008). How to predict and prevent the immunogenicity of therapeutic proteins. *Biotechnol Ann Rev 14*, 191–202.
22. Van de Weert, M., Moller, E.H. (2008). *Immunogenicity of Biopharmaceuticals: Causes, Methods to Reduce Immunogenicity, and Biosimilars*. van de Weert M, Moller EH, eds. Springer, New York, pp. 97–111.
23. Wadhwa, M., Thorpe, R. (2006). Strategies and assays for the assessment of unwanted immunogenicity. *J Immunotoxicol*, *3*, 115–121.
24. Nechansky, A. (2009). HAHA—nothing to laugh about. Measuring the immunogenicity (human anti-human antibody response) induced by humanized monoclonal antibodies applying ELISA and SPR technology. *J Pharm Biomed Anal*, *51*, 252–254.
25. Neyer, L., Hiller, J., Gish, K., et al. (2006). Confirming human antibody responses to a therapeutic monoclonal antibody using a statistical approach. *J Immunol Methods*, *315*, 80–87.

7

THE USE OF PHARMACODYNAMICS AS A SURROGATE MARKER FOR THE DETECTION OF ANTI-DRUG NEUTRALIZING ANTIBODIES

Florian Deisenhammer

7.1. INTRODUCTION

The term "neutralizing antibodies" (NAbs) describes perfectly well the dichotomy of the assessment of antibody-mediated neutralization of interferon or any other biopharmaceutical, in that measurement of both—neutralization and antibodies—cannot be done in one single assay but requires a test for antibodies and a test for neutralization as illustrated in Figure 7.1. There are many ways to test for antibodies (which is discussed in another chapter) [1] and there are probably even more ways to assess neutralization [2].

In the case of interferon, measurements of neutralization are based upon the biological pathway of this cytokine by quantification of interferon-induced markers; however, it is usually not a direct measurement of interferon itself because its half-life is very short and reasonable serum or plasma levels are not achieved in current treatment regimens [3, 4]. Other biopharmaceuticals, such as therapeutic antibodies (e.g., natalizumab), might be assessed directly by quantification of their serum or plasma levels [5].

Neutralization of interferons can be investigated in special—mostly cell-based—bioassays as discussed in other chapters but also by direct *in vivo* or

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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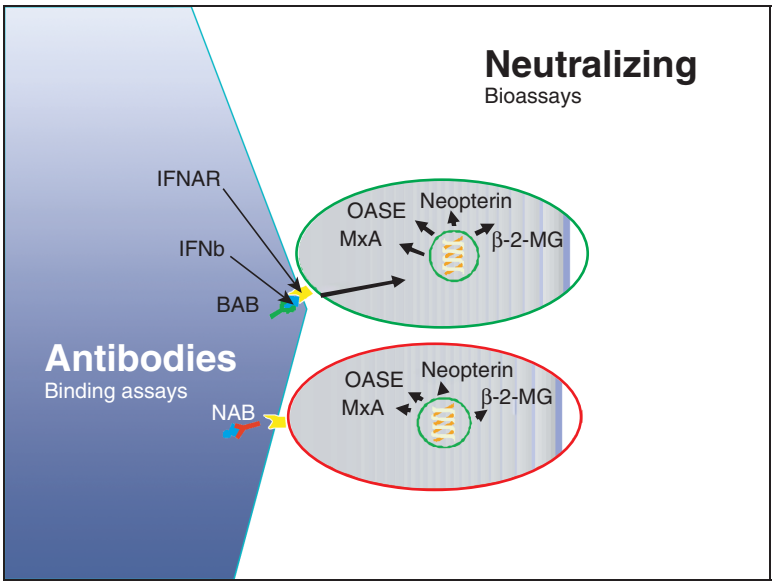

Fig. 7.1 Illustration of the dichotomy of neutralizing-antibody testing. On the left the antibody side of testing is depicted. Binding of type I interferons (IFN-α and IFN-β) to the type I interferon receptor (IFNAR) induces upregulation of several hundred gene products, such as myxovirus resistance protein (MxA), oligo-A-synthetases (OASE), neopterin, and beta-2-microglobulin (β-2-MG). Antibodies against interferon may not interfere with binding of the IFN molecule to its receptor and are then referred to as binding antibodies (BAbs). On the other hand, if antibodies block binding of interferons to the receptor, they are referred to as neutralizing antibodies (NAbs). Simple antibody tests such ELISA or Western blot are not able to distinguish between BAbs and NAbs. Therefore, for evaluation the biological effect of IFN antibodies, the bioactivity of IFN needs to be assessed (shown on right-hand side of the figure). Principally, all IFN-induced gene products may serve as outcome measure of IFN activity, but their sensitivity and specificity vary substantially. (See color insert.)

ex vivo measurement of interferon bioactivity. This chapter focuses on the latter methods.

7.2. MARKERS OF TYPE-I INTERFERON BIOACTIVITY

When interferon (IFN) is injected into a person as a therapeutic agent subcutaneously or intramuscularly, for example, as a standard therapy in multiple sclerosis (MS), there is a short period of minimally increased serum levels of IFN [3]. Maximum concentrations of 1.3 international units (IU) occur 1–2 hours after injection and are only slightly above baseline. Due to this very low assay sensitivity, laboratory monitoring of IFN therapy by direct measurement of IFN serum levels does not seem to be useful. Therefore, investigation of

IFN bioactivity must rely on other markers that are induced by type I IFNs with a much greater pharmacodynamic range in terms of peak concentrations and duration.

The number of type I IFN–induced genes is enormously high. In a recent investigation 1077 genes were found to be regulated by IFN-β at a 5% false discovery rate (FDR) level [6]. For obvious reasons this chapter will not discuss each of these single markers but will focus on some of those which have been most frequently used in the past and turned out to be valuable. The cellular type I IFN pathway is shown in Figure 7.2.

Especially in the context of the evaluation of the biological effects of NAbs, neopterin, beta-2-microglobulin (β-2-MG), oligo-A-synthetase (OAS), tumor

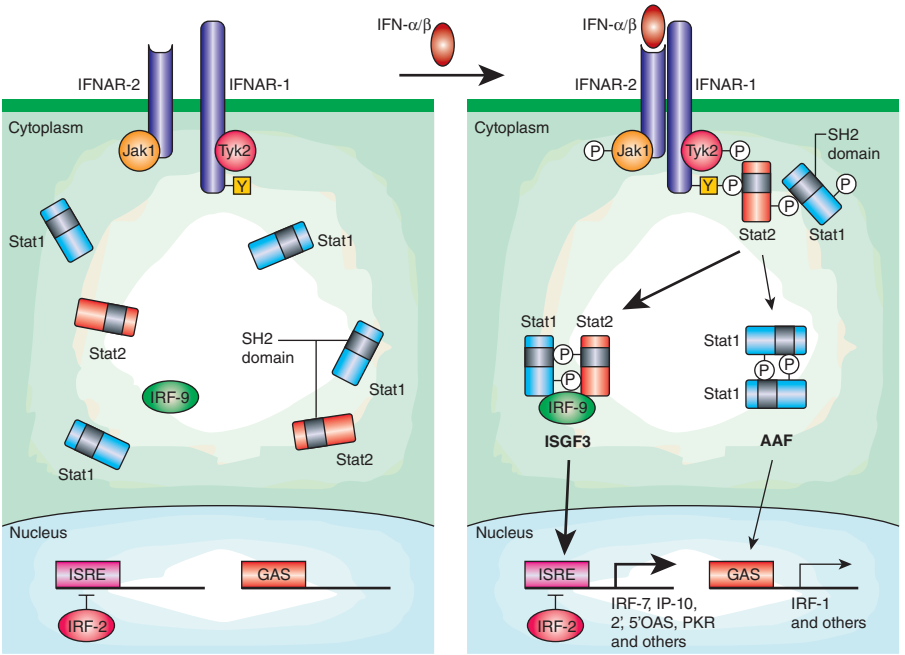

Fig. 7.2 Type I interferon transmits signals through its homologous receptor complex IFNAR, which is composed of at least two units, IFNAR-1 and IFNAR-2. IFN binding leads to the aggregation of the units and activation of the receptor-associated Tyk2 and Janus kinase (Jak1). This is followed by the tyrosine phosphorylation of STAT1 and STAT2, leading to formation of the heterotrimeric ISGF3 transcription factor, together with an IRF-family member IRF-9. In addition, a STAT1 homodimer, termed AAF22, is also formed. These transcriptional–activator complexes translocate into the nucleus and activate the interferon-stimulated response element (ISRE) or IFN-activated site (GAS), which is followed by a huge number of upregulated or downregulated genes and their respective products. [*Source: Nature Reviews Molecular Cell Biology* (2001) *2*, 378–386.] (See color insert.)

necrosis factor related apoptosis inducing ligand (TRAIL), X-linked inhibitor of apoptosis factor-1 (XAF-1), Viperin, STAT-1, PKR, s-VCAM, and Mx-proteins have been investigated on RNA as well as protein levels [7–20]. There is no proof that any of these biomarkers directly mediates the therapeutic effect of IFN-β although this cannot be entirely ruled out. Some small clinical studies showed a correlation between the levels of some of these biomarkers with the clinical response [21]. Mostly, however, they simply indicate the presence of IFN activity or the lack thereof.

Applying newer multiarray techniques to explore multiple expression patterns of IFN-induced gene activity revealed further biomarkers that might be involved in disease-regulatory mechanisms. Examples are downregulation of interleukin-8 (IL-8), or integrins such as very late activation antigen-4 (VLA-4) and lymphocyte function associated antigen-1 (LFA-1) [22, 23], and upregulation of interleukin-10 (IL-10), the chemokine CXCL-10, TRAIL and XAF-1 [15, 24]. However, as noted for other IFN biomarkers, a correlation between the level of IFN-induced activity of these gene products and the clinical response could not be consistently established. For example, TRAIL levels were reported to predict the clinical response to IFN-β [25] but this observation could not be confirmed by others [26]. In an analysis of our MS registry we could not detect a correlation between soluble TRAIL and the clinical response to IFN-β in MS patients, as well as other type I IFN biomarkers, including soluble CD80, myxovirus resistance protein (MxA), and soluble PECAM-1 (unpublished data).

7.2.1. Are Biomarkers of IFN Activity Interchangeable?

Theoretically, all IFN-induced gene products that occur downstream of the interferon-induced response element (ISRE; see Fig. 7.2) use the same activation pathway and should therefore indicate activation of IFNAR (i.e., binding of IFN to its receptor) equally. In fact, there seems to be a high correlation between various biomarkers [11, 15, 16], the difference being the levels of expression with a continuum between very low and very high concentrations. Obviously, a high level of expression is desirable but other factors need to be considered, such as the specificity for type I IFN. Although induced by type I IFNs, many markers are not entirely specific for type I IFNs, such as neopterin and β-2-MG. Both molecules are partially associated with MS disease activity and both can be induced by IFN-γ [27–29].

There are also methodological and pharmacodynamic aspects. How difficult or easy is it to detect the marker in question? Simple quantitative, semiquantitative, or qualitative methods such as enzyme-linked immunosorbent assay (ELISA) or Western blot to detect soluble molecules in serum or plasma are definitely preferable. Biomarkers that are translated to the protein level and that have a quick increase of concentration with a relatively long plateau phase are more reliable than markers that are expressed at a low level with short half-lives. A dose-dependent relationship between IFN and the biomarker is

desirable if a correlation between clinical and pharmacological response is being investigated.

Among the many IFN-induced gene products one has been extensively used and investigated, namely, the *Mx1* and *Mx2* genes translating into the Mx-proteins A and B, of which MxA is the most widely explored IFN-β biomarker in the therapy of multiple sclerosis.

7.2.2. Mx Genes and MxA Protein

The Mx proteins are members of the dynamin superfamily and occur in virtually all mammals [30]. In humans there two Mx genes, *Mx1* and *Mx2*, located on the distal part of the long arm of chromosome 21 (21q22.3) that encode the MxA and MxB proteins [31]. The MxA protein is expressed in the cell plasma, has a molecular weight of approximately 75 kDa, binds to guanosine triphosphate (GTP), and displays an intrinsic GTPase activity. By that it mediates a strong antiviral activity as part of the type I IFN response to viral infections [30, 32].

The MxA protein is specifically upregulated after application of a therapeutic type I IFN [33] but not by type II IFN (i.e., IFN-γ) and can be quantified in human whole blood by a simple sandwich ELISA technique [34]. The pair of MxA antibodies used in this assay was temporarily protected by patent and therefore unavailable. Many researchers had to circumvent these restrictions and during that time polymerase chain reaction (PCR) methods for semiquantification of MxA RNA became increasingly popular [2, 11, 35].

The difference between the ELISA and PCR methods becomes apparent on several levels.

There is a commercial MxA protein assay available, which can be bought as an ELISA kit. The direct costs of reagents for the PCR method are relatively high as well as the equipment. The ELISA procedure is simple whereas PCR methods require a higher level of laboratory expertise.

After IFN injection, the MxA protein remains stable for up to 48 hours [36]; therefore, the time of blood collection after injection hardly influences the assay results compared with The MxA RNA, which follows a steep increase and decrease of concentration with a peak at 4–12 hours after injection [17, 37] requiring very narrow time intervals between injection and blood collection to make interindividual results comparable. On the other hand, MxA protein may accumulate in patients who are treated with IFN preparations that are injected every other day or twice a week, thereby overestimating IFN bioavailability in patients on such dosage regimens [38].

A few attempts have been made to correlate the clinical response to the level of MxA expression in IFN-β-treated MS patients. In small study, Kracke and colleagues could detect higher MxA levels in clinical responders than in nonresponders [21] among NAb-negative patients, but these data were never confirmed in a similar setting. In a larger study, Malucchi et al. were able to show a correlation between MxA concentration and clinical response, but

there was a strong coincidence with NAbs in nonresponders and, therefore, the overall results were probably confounded by the presence of NAbs, although unfortunately this was not formally tested [39].

7.3. TYPE-I INTERFERON BIOMARKERS AS INDICATORS OF NEUTRALIZING ANTIBODIES

For a long time NAb testing has been a cumbersome and complex procedure relying on the detection of the antiviral properties of IFN. A typical antiviral NAb assay took 4 days and numerous steps to perform [40]. Also, NAbs against IFN and titers obtained by these assays did not mean too much to clinicians; therefore, some researchers sought to simplify the NAb issue by measuring IFN biomarkers directly in blood samples as an indirect measure of NAbs. It turned out that there is a clear negative correlation between loss of IFN-β-inducible bioactivity markers and NAb titers (Table 7.1). However, at present it is difficult to determine a clear NAb titer cut point between IFN-β response and nonresponse. The previously generally accepted cut point between NAb positivity and negativity in a single test of 20 neutralizing units (or tenfold reduction units) does not indicate a complete loss of IFN-β bioactivity. As the number of reports increased, the grey zone between clear-cut NAb negativity and positivity became wider and now appears to lie between 20 and 600 NU (Table 7.1). One of the most important reasons for this wide range of cutoff points is the use of different antigens in the NAb assay. Testing one single sample against IFN-β1a results in a threefold to sixfold higher NAb titer than against IFN-β1b. This is due to the higher absolute amount of IFN protein load in IFN-β1b at a comparable unitage, leading to the binding of more antibodies and consequently to lower calculated NAb titers [41].

Although there is a highly significant correlation between various bioactivity markers, some appear to be less sensitive and specific to distinguish between NAb negativity and positivity, contributing to different NAb cut points [15, 16, 18]. MxA (protein and RNA) has been a very frequently used biomarker because it has good pharmacodynamic properties with a high sensitivity and specificity, and it can be easily measured as described in the previous section. Also, the between-study variation of NAb titers indicating a loss of MxA induction appears to be lower than using other biomarkers with cutoff titers between 20 and 100 NU. There were concerns as to whether MxA would stand for a complete loss of IFN-β bioactivity in NAb-positive patients or if there were other pathways that might still be intact. Therefore, a great variety of IFN biomarkers were explored and none was found to be preserved when there was no MxA response [14–19, 42]. More recently, multiarray analyses of the IFN-β response could confirm this on a broad level of essentially all IFN-induced genes (Fig. 7.3) [6, 37]. It can be concluded that one biomarker stands for all others with respect to the biological IFN-β response and the most sensitive and specific—which is probably MxA—can be used for testing.

TABLE 7.1 How Type I IFN Biomarkers Are Related to NAb Titers

Study	Marker	Level of Expression	Ag in NAb Assay	NAb Titre in Partial Biomarker Response	NAb Titre in Strongly Reduced / No Biomarker Response
Rudick et al. 1998 [7]	Neopterin	Protein	IFN-β1a	5–19 NU	≥20 NU
	β-2-MG			–	≥5 NU
Deisenhammer et al. 1999 [8]	MxA	Protein	IFN-β1b	1–19 NU	≥ 20 NU
Cook et al. 2001 [9]	Neopterin	Protein	IFN-β1a	< 60 NU	≥ 60 NU
			IFN-β1b	–	≥ 60 NU
Vallittu et al. 2002 [49]	MxA	Protein	IFN-β1a	40–160 NU	> 160 NU
Pachner et al. 2004 [11]	MxA	RNA	IFN-β1a/b	– [b]	> 200 NU
	OAS			–	> 200 NU
Bertolotto et al. 2003 [12]	MxA	RNA	IFN-β1a/b	20–44 NU	≥ 45 NU
Gilli et al. 2004 [13]	MxA[a]	RNA	IFN-β1a/b	–	≥ 20 NU
Sorensen et al. 2006 [14]	Neopterin	Protein	IFN-β1a/b	20–79% NC	≥ 80% NC
	β-2-MG			20–79% NC	≥ 80% NC
Gilli et al. 2006 [15]	MxA	RNA	IFN-β1a/b	≥ 20 NU	Not determined
	TRAIL			≥ 20 NU	
	XAF-1			≥ 20 NU	
Pachner et al. 2006 [16]	MxA	RNA	IFN-β1a/b	20–100 NU	>100 NU
	Viperin			20–100 NU	>100 NU
	OAS			20–100 NU	>100 NU
Santos et al. 2006 [17]	MxA	RNA	IFN-β1a	1–19 NU	Not determined
	MxB			1–19 NU	
	STAT-1			1–19 NU	
	TRAIL			1–19 NU	
	β-2-MG			1–19 NU	
Scagnolari et al. 2007 [18]	Neopterin	Protein	IFN-β1b	20–200 NU	> 200 NU
	β-2-MG	Protein		–	≥ 20 NU
	MxA	RNA		20–200 NU	>200 NU
	PKR	RNA		20–200 NU	> 200 NU
Sominanda et al. 2008 [19]	MxA	RNA	IFN-β1a	150–600 NU	> 600 NU
	TRAIL			150–600 NU	> 600 NU

Source: *CNS Drugs* (2009) *23*, 379–396.

Depending on the marker, the method of NAb detection, and the assay format the markers of type I IFN bioactivity are not detectable at NAb titers above 5–600 NU.

[a]Other biomarkers were investigated (metalloproteinases and inhibitors). No immediate response, but a delayed response to IFN-β could be detected.

[b]Only titers greater than 200 NU were detected in this study.

Abbreviations: β-2-MG, beta-2-microglobulin; OAS, oligo-A-synthetase; MxA, *Myxovirus* resistence; TRAIL, tumor necrosis factor related apoptosis inducing ligand; XAF-1, X-linked inhibitor of apoptosis factor-1; NU, neutralizing units (as calculated by the Kawade formula) [50]; NC, neutralizing capacity. This method uses a fixed amount of antibodies and titrates the antigen [51].

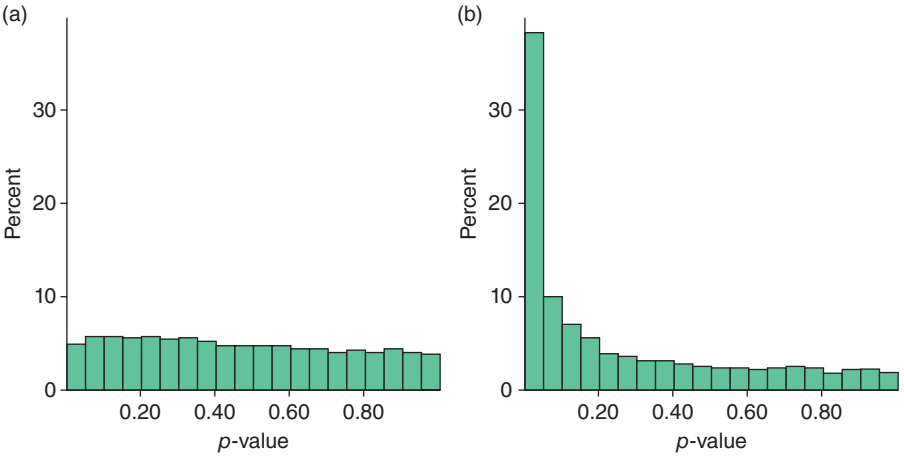

Fig. 7.3 The *p*-value distribution reflecting interferon IFN-β-regulated genes: *p*-values from 5593 paired *t*-tests of detectable genes on Affymetrix. The *p*-values are grouped in intervals of 0.05. (A) Neutralizing antibody (NAb)-positive patients. (B) NAb-negative patients. [*Source: Neurology* (2009) *73,* 372–377.]

7.3.1. How Can Type-I IFN Biomarkers Contribute to the Procedure of NAb Testing?

As discussed earlier, NAb testing can be done at several levels and rarely does one single test provide enough information for clinical decision making. The conventional cell-based NAb assay is very valuable but there are some caveats with such assays. Although a high NAb titer as measured by a NAb assay will be mostly associated with a complete loss of IFN bioactivity, sometimes this result might be false specifically when soluble IFN-receptors are present [43]. In such cases IFN biomarker testing can provide additional information.

In patients with intermediate NAB titers –a gray zone that is not finally determined but probably ranges from 20 to 600 NU –biomarkers can be very useful because individual cutoff points vary substantially.

In contrast, what are the advantages and disadvantages of primary IFN biomarker testing without determining NAb titers by conventional NAb assays? There are some drawbacks. A lack of IFN bioactivity may not necessarily indicate the presence of NAbs. Some patients forget or occasionally refuse to inject [44], which of course would not result in measurable IFN effects, leading to a false positive test result. Accumulation of biomarkers in high-frequency IFN-β preparations or too long time intervals between injection and blood collection can again influence test outcomes. Furthermore, once the NAb titer is higher than the cutoff point for bioactivity there will be no further decrease of biomarker concentrations. NAb titers, however, provide important information about the persistency of antibodies [45, 46]. The higher the titer, the more likely NAbs persist and then are associated with a perma-

nent loss of treatment effects [47]. When using IFN biomarkers for the evaluation of NAbs one must consider all these aspects. The advantages are the relative ease of the assay and the information about the bioavailability. Some investigators simply rely on BAb and biomarker testing without performing NAb tests [48]. High NAb titers correlate well with BAb titers [1] and information about IFN bioactivity makes clinicians more confident regarding therapeutic decisions, especially if stopping IFN treatment is considered.

To get reliable and comparable results one would suggest patients to be off the drug for some days, ideally for one week to make sure that baseline bioactivity levels are zero. To estimate the individual IFN response, preinjection biomarker levels should be determined and the difference to postinjection concentrations can be calculated, of course requiring standardized time intervals between injection and blood collection. This has some procedural disadvantages compared with a standard NAb test, which can essentially be done any time.

7.4. SUMMARY

When injected in the body, type I interferons upregulate or downregulate a huge number of genes and their products. Neutralizing antibodies against interferons block receptor binding and, consequently, lead to a lack of this specific gene induction. Therefore, such biomarkers can be used as surrogates for the presence of NAbs. The most extensively investigated and probably most sensitive and specific marker of type I interferon bioactivity is the *Mx1* gene translating into the MxA protein. MxA is coexpressed with all other known type I interferon biomarkers and can be representatively used for IFN bioactivity. In combination with interferon binding antibody titers, MxA levels might provide even more information than NAb titers alone.

REFERENCES

1. Gneiss, C., Brugger, M., Millonig, A., Fogdell-Hahn, A., Rudzki, D., Hillert, J., et al. (2008). Comparative study of four different assays for the detection of binding antibodies against interferon-beta. *Mult Scler*, *14*, 830–836.
2. Deisenhammer, F., Schellekens, H., Bertolotto, A. (2004). Measurement of neutralizing antibodies to interferon beta in patients with multiple sclerosis. *J Neurol*, *251* (Suppl. 2), II31-II39.
3. Salmon, P., Le Cotonnec, J.Y., Galazka, A., Abdul-Ahad, A., Darragh, A. (1996). Pharmacokinetics and pharmacodynamics of recombinant human interferon-beta in healthy male volunteers. *J Interferon Cytokine Res*, *16*, 759–764.
4. Williams, B.R. (1991). Transcriptional regulation of interferon-stimulated genes. *Eur J Biochem*, *200*, 1–11.

5. Calabresi, P.A., Giovannoni, G., Confavreux, C., Galetta, S.L., Havrdova, E., Hutchinson, M., et al. (2007). The incidence and significance of anti-natalizumab antibodies: results from AFFIRM and SENTINEL. *Neurology*, *69*, 1391–1403.
6. Hesse, D., Sellebjerg, F., Sorensen, P.S. (2009). Absence of MxA induction by interferon beta in patients with MS reflects complete loss of bioactivity. *Neurology*, *73*, 372–377.
7. Rudick, R.A., Simonian, N.A., Alam, J.A., Campion, M., Scaramucci, J.O., Jones, W., et al. (1998). Incidence and significance of neutralizing antibodies to interferon beta-1a in multiple sclerosis. Multiple Sclerosis Collaborative Research Group (MSCRG) [see comments]. *Neurology*, *50*, 1266–1272.
8. Deisenhammer, F., Reindl, M., Harvey, J., Gasse, T., Dilitz, E., Berger, T. (1999). Bioavailability of interferon beta 1b in MS patients with and without neutralizing antibodies. *Neurology*, *52*, 1239–1243.
9. Cook, S.D., Quinless, J.R., Jotkowitz, A., Beaton, P. (2001). Serum IFN neutralizing antibodies and neopterin levels in a cross-section of MS patients. *Neurology*, *57*, 1080–1084.
10. Prummer, O. (1997). Treatment-induced antibodies to interleukin-2. *Biotherapy*, *10*, 15–24.
11. Pachner, A.R., Narayan, K., Price, N., Hurd, M., Dail, D. (2004). MxA gene expression analysis as an interferon-beta bioactivity measurement in patients with multiple sclerosis and the identification of antibody-mediated decreased bioactivity. *Mol Diagn*, *7*, 17–25.
12. Bertolotto, A., Gilli, F., Sala, A., Capobianco, M., Malucchi, S., Milano, E., et al. (2003). Persistent neutralizing antibodies abolish the interferon beta bioavailability in MS patients. *Neurology*, *60*, 634–639.
13. Gilli, F., Bertolotto, A., Sala, A., Hoffmann, F., Capobianco, M., Malucchi, S., et al. (2004). Neutralizing antibodies against IFN-beta in multiple sclerosis: antagonization of IFN-beta mediated suppression of MMPs. *Brain*, *127*, 259–268.
14. Sorensen, P.S., Tscherning, T., Mathiesen, H.K., Langkilde, A.R., Ross, C., Ravnborg, M., et al. (2006). Neutralizing antibodies hamper IFNbeta bioactivity and treatment effect on MRI in patients with MS. *Neurology*, *67*, 1681–1683.
15. Gilli, F., Marnetto. F., Caldano, M., Sala, A., Malucchi, S., Capobianco, M., et al. (2006). Biological markers of interferon-beta therapy: comparison among interferon-stimulated genes MxA, TRAIL and XAF-1. *Mult Scler*, *12*, 47–57.
16. Pachner, A.R., Narayan, K., Pak, E. (2006). Multiplex analysis of expression of three IFNbeta-induced genes in antibody-positive MS patients. *Neurology*, *66*, 444–446.
17. Santos, R., Weinstock-Guttman, B., Tamano-Blanco, M., Badgett, D., Zivadinov, R., Justinger, T., et al. (2006). Dynamics of interferon-beta modulated mRNA biomarkers in multiple sclerosis patients with anti-interferon-beta neutralizing antibodies. *J Neuroimmunol*, *176*, 125–133.
18. Scagnolari, C., Duda. P., Bagnato, F., De Vito, G., Alberelli, A., Lavolpe. V., et al. (2007). Pharmacodynamics of interferon beta in multiple sclerosis patients with or without serum neutralizing antibodies. *J Neurol*, *254*, 597–604.
19. Sominanda, A., Hillert, J., Fogdell-Hahn, A. (2008). In vivo bioactivity of interferon-beta in multiple sclerosis patients with neutralising antibodies is titre-dependent. *J Neurol Neurosurg Psychiatry*, *79*, 57–62.

20. Deisenhammer, F. (2009). Neutralising antibodies to immunological treatments for multiple sclerosis: prevalence and impact on outcomes. *CNS Drugs*, *23*, 379–396.
21. Kracke, A., Von Wussow. P., Al-Masri, A.N., Dalley, G., Windhagen, A., Heidenreich, F. (2000). Mx proteins in blood leukocytes for monitoring interferon beta-1b therapy in patients with MS. *Neurology*, *54*, 193–199.
22. Sturzebecher, S., Wandinger, K.P., Rosenwald, A., Sathyamoorthy, M., Tzou, A., Mattar, P., et al. (2003). Expression profiling identifies responder and non-responder phenotypes to interferon-beta in multiple sclerosis. *Brain*, *126*, 1419–1429.
23. Muraro, P.A., Liberati, L., Bonanni, L., Pantalone, A., Caporale, C.M., Iarlori, C., et al. (2004). Decreased integrin gene expression in patients with MS responding to interferon-beta treatment. *J Neuroimmunol*, *150*, 123–131.
24. Hesse, D., Frederiksen, J.L., Koch-Henriksen, N., Schreiber, K., Stenager, E., Heltberg, A., et al. (2009). Methylprednisolone does not restore biological response in multiple sclerosis patients with neutralizing antibodies against interferon-beta. *Eur J Neurol*, *16*, 43–47.
25. Wandinger, K.P., Lunemann, J.D., Wengert, O., Bellmann-Strobl, J., Aktas, O., Weber, A., et al. (2003). TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis. *Lancet*, *361*, 2036–2043.
26. Buttmann, M., Merzyn, C., Hofstetter, H.H., Rieckmann, P. (2007). TRAIL, CXCL10 and CCL2 plasma levels during long-term interferon-beta treatment of patients with multiple sclerosis correlate with flu-like adverse effects but do not predict therapeutic response. *J Neuroimmunol*, *190*, 170–176.
27. Giovannoni, G., Lai, M., Kidd, D., Thorpe, J., Miller, D., Thompson, A., et al. (1997). Daily urinary neopterin excretion as an immunological marker of disease activity in multiple sclerosis. *Brain*, *120*, 1–13.
28. Vraetz, T., Ittel, T.H., van Mackelenbergh, M.G., Heinrich, P.C., Sieberth, H.G., Graeve, L. (1999). Regulation of beta2-microglobulin expression in different human cell lines by proinflammatory cytokines. *Nephrol Dial Transplant*, *14*, 2137–2143.
29. Huber, C., Batchelor, J.R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., et al. (1984). Immune response–associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. *J Exp Med*, *160*, 310–316.
30. Horisberger, M.A. (1995). Interferons, Mx genes, and resistance to influenza virus. *Am J Respir Crit Care Med*, *152*, S67–S71.
31. Horisberger, M.A., Wathelet, M., Szpirer, J., Szpirer, C., Islam, Q., Levan, G., et al. (1988). cDNA cloning and assignment to chromosome 21 of IFI-78K gene, the human equivalent of murine Mx gene. *Somat Cell Mol Genet*, *14*, 123–131.
32. Roers, A., Hochkeppel, H.K., Horisberger, M.A., Hovanessian, A., Haller, O. (1994). MxA gene expression after live virus vaccination: a sensitive marker for endogenous type I interferon. *J Infect Dis*, *169*, 807–813.
33. Von Wussow, P., Jakschies, D., Hochkeppel, H.K., Fibich, C., Penner, L., Deicher, H. (1990). The human intracellular Mx-homologous protein is specifically induced by type I interferons. *Eur J Immunol*, *20*, 2015–2019.
34. Towbin, H., Schmitz, A., Jakschies, D., Von Wussow, P., Horisberger, M.A. (1992). A whole blood immunoassay for the interferon-inducible human Mx protein. *J Interferon Res*, *12*, 67–74.

35. Bertolotto, A., Gilli, F., Sala, A., Audano, L., Castello, A., Magliola, U., et al. (2001). Evaluation of bioavailability of three types of IFNbeta in multiple sclerosis patients by a new quantitative-competitive-PCR method for MxA quantification. *J Immunol Methods*, *256*, 141–152.
36. Deisenhammer, F., Mayringer, I., Harvey, J., Dilitz, E., Gasse, T., Stadlbauer, D., et al. (2000). A comparative study of the relative bioavailability of different interferon beta preparations. *Neurology*, *54*, 2055–2060.
37. Sellebjerg, F., Krakauer, M., Hesse, D., Ryder, L.P., Alsing, I., Jensen, P.E., et al. (2009). Identification of new sensitive biomarkers for the in vivo response to interferon-beta treatment in multiple sclerosis using DNA-array evaluation. *Eur J Neurol*, *16*, 1291–1298.
38. Williams, G.J., Witt, P.L. (1998). Comparative study of the pharmacodynamic and pharmacologic effects of Betaseron and Avonex. *J Interferon Cytokine Res*, *18*, 967–975.
39. Malucchi, S., Gilli, F., Caldano, M., Marnetto, F., Valentino, P., Granieri, L., et al. (2008). Predictive markers for response to interferon therapy in patients with multiple sclerosis. *Neurology*, *70*, 1119–1127.
40. Kawade, Y. (1986). Quantitation of neutralization of interferon by antibody. *Methods Enzymol*, *119*, 558–573.
41. Bertolotto, A., Malucchi, S., Milano, E., Castello, A., Capobianco, M., Mutani, R. (2000). Interferon beta neutralizing antibodies in multiple sclerosis: neutralizing activity and cross-reactivity with three different preparations. *Immunopharmacol*, *48*, 95–100.
42. Gandhi, K.S., McKay, F.C., Schibeci, S.D., Arthur, J.W., Heard, R.N., Stewart, G.J., et al. (2008). BAFF is a biological response marker to IFN-beta treatment in multiple sclerosis. *J Interferon Cytokine Res*, *28*, 529–539.
43. Gilli, F., Valentino, P., Caldano, M., Granieri, L., Capobianco, M., Malucchi, S., et al. (2008). Expression and regulation of IFNalpha/beta receptor in IFNbeta-treated patients with multiple sclerosis. *Neurology*, *71*, 1940–1947.
44. Twork, S., Nippert, I., Scherer, P., Haas, J., Pohlau, D., Kugler, J. (2007). Immunomodulating drugs in multiple sclerosis: compliance, satisfaction and adverse effects evaluation in a German multiple sclerosis population. *Curr Med Res Opin*, *23*, 1209–1215.
45. Sorensen, P.S., Koch-Henriksen, N., Ross, C., Clemmesen, K.M., Bendtzen, K., the Danish Multiple Sclerosis Study Group. (2005). Appearance and disappearance of neutralizing antibodies during interferon-beta therapy. *Neurology*, *65*, 33–39.
46. Gneiss, C., Reindl, M., Lutterotti, A., Ehling, R., Egg, R., Khalil, M., et al. (2004). Interferon-beta: the neutralizing antibody (NAb) titre predicts reversion to NAb negativity. *Mult Scler*, *10*, 507–510.
47. Polman, C., Kappos, L., White, R., Dahlke, F., Beckmann, K., Pozzilli, C., et al. (2003). Neutralizing antibodies during treatment of secondary progressive MS with interferon beta-1b. *Neurology*, *60*, 37–43.
48. Hoffmann, S., Cepok, S., Grummel, V., Lehmann-Horn, K., Hackermueller, J., Stadler, P.F., et al. (2008). HLA-DRB1*0401 and HLA-DRB1*0408 are strongly associated with the development of antibodies against interferon-beta therapy in multiple sclerosis. *Am J Hum Genet*, *83*, 219–227.

49. Vallittu, A.M., Halminen, M., Peltoniemi, J., Ilonen, J., Julkunen, I., Salmi, A., et al. (2002). Neutralizing antibodies reduce MxA protein induction in interferon-beta-1a-treated MS patients. *Neurology*, *58*, 1786–1790.

50. Kawade, Y. (1980). An analysis of neutralization reaction of interferon by antibody: a proposal on the expression of neutralization titer. *J Interferon Res*, *1*, 61–70.

51. Ross, C., Clemmesen, K.M., Svenson, M., Sorensen, P.S., Koch-Henriksen, N., Skovgaard, G.L., et al. (2000). Immunogenicity of interferon-beta in multiple sclerosis patients: influence of preparation, dosage, dose frequency, and route of administration. Danish Multiple Sclerosis Study Group. *Ann Neurol*, *48*, 706–712.

8

CELL-BASED ASSAYS FOR THE DETECTION OF NEUTRALIZING ANTIBODIES TO INTERFERON BETA (IFN-β) AND TUMOR NECROSIS FACTOR ALPHA (TNF-α) INHIBITORS

Anthony Meager

8.1. INTRODUCTION

Several human cytokines, including interferon alpha (IFN-α), interferon beta (IFN-β), interleukin-2 (IL-2), granulocyte colony-stimulating factor (G-CSF), and granulocyte–macrophage colony-stimulating factor (GM-CSF), are in use clinically as therapeutic agents. These are mainly manufactured by recombinant DNA technological processes and invariably exhibit some, albeit usually minor, structural differences from cytokines produced naturally by human cells *in vivo*. In addition, a growing number of inhibitors of cytokines are finding clinical uses, particularly for treating arthritis and other inflammatory diseases. Here, the majority of cytokine inhibitor products are monoclonal antibodies, though some soluble cytokine receptors have also been developed. The monoclonal antibodies include murine, human, and chimeric murine–human immunoglobulins, which mostly differ structurally from native human ones.

Following the introduction of the clinical use of therapeutic cytokines and cytokine inhibitors, significant numbers of patients have developed

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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antitherapeutic antibodies (ATAs) to the administered cytokine or cytokine inhibitor. The level and type, whether non-neutralizing (binding) or neutralizing, of ATAs developed appears to depend on several factors, including the extent of any structural differences between the manufactured product and its natural counterpart, nature of excipients in the product formulation, degree of product aggregation and/or oxidation, dose, route and frequency of administration, and immune status of the patient treated. There is some evidence that immunogenicity is product-related, but in most cases immunogenicity cannot be predicted from preclinical studies. A variable number of patients develop ATAs; in only a proportion of these do the ATAs become clinically significant for continuation of therapy and/or the disease's course. However, for such patients, product immunogenicity has an unwanted and sometimes a major impact on product efficacy and safety. Thus, if possible, it is important to monitor all patients undergoing treatment with a particular therapeutic cytokine or cytokine antagonist for the development of specific ATAs. Since neutralizing ATAs, rather than non-neutralizing ATAs, are more likely to exert undesirable biological and clinical sequelae, tests or assays for neutralizing ATAs are required for monitoring their development. This normally means employing cell-based cytokine activity assays to detect and quantify neutralizing ATAs.

This review is focused first on cell-based assays for detection and quantification of IFN-β to illustrate the wide diversity of approaches in this field. Therapeutic IFN-β products are widely used in the treatment of relapsing–remitting multiple sclerosis. In this case, many useful, reproducible methods have been developed to detect neutralizing antibodies (NAbs) to IFN-β and this review aims to compare their implementation and effectiveness. In contrast to the maturity of anti-IFN-β NAb assays, assay methodology to detect neutralizing ATAs to monoclonal antibody (mAb) therapeutics is in its infancy and is more problematic. Due to high dose levels, neutralizing ATAs are likely to be present in immunoglobulin complexes with the mAb therapeutic and thus require separation methods to be applied before they can be reliably detected. This review will take anti-TNF-α therapeutics as an example to illustrate potential approaches to detecting and quantifying NAbs to them.

8.2. INTERFERON-BETA TREATMENT OF MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a debilitating, incurable neurological disease of unknown cause that affects over one million persons worldwide. Patients suffer increasing disturbances of vision, co-ordination, sensation, gait, and endurance, though the clinical course is unpredictable. Immunopathology takes the form of active lesions of neurone demyelination in the brain, for which the use of magnetic resonance imaging plays a critical role in diagnosis and management [1–3]. In the initial stage, recurrent attacks (exacerbations) occur infrequently, with lengthy disease-free intervals. This form of the

disease is known as relapsing–remitting MS (RRMS), but this is often followed by a more insidious progression of functional impairment known as secondary progressive MS (SPMS) leading to increasing morbidity and mortality [1–3].

Data from clinical studies have indicated that IFN-β can reduce the frequency and severity of exacerbations, slow disease progression, and improve the quality of life for MS patients [4–6]. Therefore, recombinant IFN-β treatment has been approved and widely accepted for patients with either RMMS or SPMS, though efficacy for the latter is weak. Although IFN-β is specified by a single gene, the IFN-β protein has one *N*-glycosylation site at Asn-80, and N-linked oligosaccharides, primarily of the biantennary complex type, are attached to this site in IFN-β secreted from human cells [7]. The carbohydrate composition varies according to producer cell type. Thus, when recombinant IFN-β is produced in mammalian cells, for example, Chinese hamster ovary (CHO) cells, it will bear oligosaccharides specified by the glycosylation machinery of CHO cells. They are distinct from those specified by human cells [8, 9]. Two clinically approved, innovator IFN-β products are manufactured from CHO cells: they are generically classified as IFN-β1a. In contrast, recombinant IFN-β produced by *Escherichia coli* lacks carbohydrate and is prone to aggregation due to the formation of intermolecular disulfide bonds [10]. However, replacement of the unpaired Cys residue at position 17 with Ser has resulted in a more stable product, now classified as IFN-β1b, but with markedly lower specific activity than IFN-β1a [10–12].

Currently, three clinically approved, innovator IFN-β products varying in structure and formulation have been developed, manufactured, and marketed for treating patients with RRMS. These are two IFN-β1a products, Avonex (Biogen-IDEC) and Rebif (Merck Serono), which are formulated differently, and one IFN-β1b product, Betaseron (or Betaferon depending on the country in which it is being marketed; Schering AG). However, following the expiration of the patent on IFN-β production, several "subsequent-entry" or "biosimilar" IFN-β products, mostly of the IFN-β1a variety, have been developed. They are being manufactured and marketed principally in economically constrained countries, often in competition with the approved, innovator IFN-β products.

A consequence of recombinant IFN-β treatment of patients with RRMS or SPMS is the variable development of antibodies to the therapeutic IFN-β1a and IFN-β-1b products [6, 13–15]. Such antibodies are usually of the immunoglobulin G (IgG) class and can be either non-neutralizing antibodies—often erroneously termed binding antibodies (BAbs)—or neutralizing antibodies (NAbs) [16–20]. The former are more prevalent than NAbs, but simply bind to IFN-β molecules without apparently affecting their activity. The latter, however, bind IFN-β molecules in a way that sterically inhibits attachment to cell surface type I IFN receptors, thus inhibiting and ultimately abrogating their activity. At least two neutralizing epitopes, which span the linear sequences of amino acid residues 1–12 in the N-terminal portion and 151–162 at the C-terminus, have been characterized [21].

8.3. CELL-BASED ASSAYS FOR DETECTING NEUTRALIZING ANTIBODIES TO IFN-β

The type I IFN family, to which IFN-β belongs, has several well-characterized biological activities. These include antiviral, antiproliferative, differentiating, and immunomodulatory activities [22, 23]. Each and any of them can form the basis of assay methods to measure the potency of type I IFN preparations. Historically, antiviral assays (AVAs) where IFNs inhibit the replication of a virus in a dose-dependent fashion were first developed for potency estimations. Since type I IFN receptors are widely distributed among cell types and cell lines, and there are several different IFN-sensitive viruses to choose from, there are many "cell–virus combinations" suitable for AVAs. Additionally, different aspects of viral replication, including RNA and protein synthesis, cytopathic effect, and production of progeny virus, are quantifiable. Thus, a wide variety of AVAs with differing cell–virus combinations and readouts has been developed and validated for measuring IFN potency [23, 24]. In contrast, antiproliferative assays in which IFNs inhibit cell division are only rarely used. Not only are they less sensitive but also are likely to take 3–7 days to complete [22, 23].

Although AVAs remain commonly used today, they too are time-consuming and often cumbersome or laborious to perform. Since safety concerns or the lack of appropriate containment facilities may prevent the performance of AVAs in some institutions, researchers have sought other, alternative, "nonvirus" assays to quantify IFNs. Much work in this area has centered on the dose-dependent upregulation of IFN-inducible proteins. For example, expression of the IFN-inducible GTPase MxA, which is measurable in an enzyme-linked immunosorbent assay (ELISA), is dependent on IFN concentration and thus can be utilized for an IFN assay [25]. However, this again, like AVA, is a two-stage assay and simpler assays based on inducible protein expression have been developed. These involve ligation of a "foreign" gene, such as one that encodes a "measurable" enzyme (e.g., firefly luciferase or alkaline phosphatase) to the promoter region of an IFN-inducible gene followed by plasmid transfection into a suitably IFN-responsive cell line [22, 23]. Establishment of stably transfected cell lines harboring the IFN-inducible promoter–enzyme gene construct then permits development of what have been dubbed "reporter gene assays (RGAs)." In these assays IFN concentration or potency is directly related to IFN-induced enzymatic activity, which can be measured by simple addition of enzyme substrate, often within hours of IFN stimulation, thus avoiding the need for a second assay stage. The major drawback of RGAs is that currently most of the genetically modified cell lines upon which they rely are not available commercially. However, at least one manufacturer is addressing this shortfall by supplying RGA kits containing division-arrested frozen cells carrying a luciferase reporter gene [26].

Going one step closer to the point of IFN stimulation, it has recently been possible to construct IFN assays based on sensitive quantitative reverse

transcription–polymerase chain reaction (qPCR) technology to determine levels of specific IFN-induced mRNA, for example, MxA mRNA, 6–16 mRNA [20, 27, 28]. Such assays require relatively short incubation periods following addition of IFN and with modern qPCR methods can be completed within a single day. If branched DNA technology is used, gene expression can be measured without the requirement for RNA extraction and cDNA synthesis, thus increasing the potential for high-throughput applications [28]. Finally, the elucidation of early phosphorylated intermediates in IFN signal transduction pathways might also lead to development of additional novel bioassays [23]. Examples of the diversity of assays now used to measure anti-IFN NAbs are listed in Table 8.1.

While all cell-based assays can be validated and calibrated for quantifying IFN potency by inclusion of appropriate World Health Organization (WHO) International Standards (IS) for IFNs, the same is not true when they are used to detect and measure anti-IFN NAbs. Neutralizing antibodies neutralize biological activity of IFNs because they bind epitopes on IFN molecules that sterically prevent them from contacting their cell surface receptors. The neutralizing capacity or strength is dependent on several factors, including ability of antibodies to bind, strength of their binding (intrinsic and functional affinities), immunoglobulin class and type, and epitope location on the surface of an IFN molecule. Thus, a wide diversity of NAbs to IFN molecules exists. This diversity increases further due to structural variations, as is evident for therapeutic IFN-β products. The inconsistency of composition of IFN-specific NAbs from patients undergoing IFN therapy renders impossible the designation of reference standards for anti-IFN NAbs. Although reference preparations of human anti-IFN-α and -β NAbs are available, and endorsed by the WHO, they are intended only for monitoring the sensitivity of cell-based assays [29]. They cannot be used in the same way as the WHO IS for IFN ligands to calibrate assays for potency determinations [23]. Thus, neutralizing activity cannot be expressed in international (reference) units.

The wide spectrum of cell-based assays has in theory the potential to provide analysts with the means to accurately measure NAbs to IFNs (Table 8.1). However, this broad choice together with variable experimental conditions and inharmonious methods for assessing and calculating neutralizing activity has already led to wide variations in the reporting of NAb titers. The latter may have a serious impact on the interpretation of results regarding clinical relevance of NAbs. Nevertheless, certain methodological elements are common to most approaches for measuring NAbs. First, the majority of assays are based on the "constant IFN method" [18]. Here dilutions of test sample—a serum from a patient or polyclonal anti-IFN immunoglobulin preparation—are mixed with a fixed concentration of IFN and incubated together for 1–2 hours to allow neutralization to take place. The mixtures are then applied to the IFN-sensitive cells and the assays are processed as if IFN were being tested. In practice, the concentration of IFN is usually set by previously informed knowledge of its potency in the assay of choice to be approximately

TABLE 8.1 Assays Used to Determine Anti-IFN-β NAb Titers

Assay Type	Cell System (Virus)	Measurement	References
Antiviral assay	2D9 human glioblastoma (EMCV)	Cytopathic effect reduction (CPER)	24
	A549 human lung adenocarcinoma (EMCV)	CPER	27, 35, 36
	HuH7 human hepatoma (VSV)	CPER	26
Bioimmunoassay	A549 human lung adenocarcinoma	MxA protein measured by ELISA	15, 25, 27, 36
Reporter gene assay	Human embryonic kidney 293P transfected with ISRE-secreted alkaline phosphatase (SEAP)	Alkaline phosphatase activity (absorbance)	22, 23, 104
	U937 human promonocytic leukemia transfected with ISRE-luciferase	Luciferase activity (luminescence)	26
	HT-1080 human fibrosarcoma (clone HL116) transfected with IFN-inducible promoter-luciferase	Luciferase activity (luminescence)	35
	HeLa human cervical carcinoma transfected with Mx2 promoter-luciferase	Luciferase activity (luminescence)	105
qPCR assay	A549 human lung adenocarcinoma	Type I IFN-inducible 6–16 mRNA expression	28
	A549 human lung adenocarcinoma	Type I IFN-inducible MxA mRNA expression	27

20 laboratory units (LU) per milliliter, reducing to 10 LU/mL when mixed with diluted test sample. Once the assays are processed the dilution of test sample at which IFN potency is diminished from 10 LU/mL to 1 LU/ml is evaluated and forms the basis of NAb titer estimation. While there are several ways in which NAb titers can be calculated, the preferred approach, at least for antiviral assays, is based on a formula provided by theoretical analysis of antibody–IFN interaction, the model of which was first proposed by Kawade [30, 31] and expanded by Grossberg, Kawade, and others later [18, 32, 33]. Fuller details of this model and method of NAb titer calculation are provided by Grossberg and Kawade [18] and elsewhere in this book.

A potential drawback of the constant-IFN method is that its sensitivity is limited by the lowest serum dilution that can be made without undue adverse effects being manifested in the cells used for assay. Many sera have growth-promoting activity and/or occasionally cytotoxic effects, which can affect assay performance. In general, serum dilutions below 1/10 are not feasible in AVA. To assess sera that may have very low NAb titers, Grossberg and others [34] have advocated a "constant antibody" method be utilized. In this case, increasing concentrations of IFN are mixed with a fixed serum dilution, for example, 1/10, to determine which IFN concentration generates the 1 LU/mL endpoint of the assay. Dilution of a serum with high NAb titer to generate preparations with low titers ranging from 1 to 20 is useful for estimating the sensitivity of assays for this purpose. NAb titer is calculated using the same formula as for the constant-IFN method. More detailed consideration is to be found in Grossberg and Kawade [18] and elsewhere in this book.

Most AVAs are exquisitely sensitive to IFNs, particularly to IFN-β, and will detect concentrations of 10 pg/mL or less. Other nonvirus assays are mostly less sensitive. Differences in assay sensitivity should in theory have little impact on NAb titer because, if the constant-IFN method is used, the IFN concentration is adjusted to 10 LU/mL for each assay. In the "rare" studies where two or more assays of the same sera have been compared side by side, the NAb titers derived from each assay were in broad agreement [20, 27, 28, 35, 36]. In this laboratory, the results of an AVA have been compared with those of an RGA based on IFN-induced secreted alkaline phosphatase for serum samples from two RRMS patients receiving IFN-β1a over an extended period of time. Both assays were incubated with the same serum dilutions mixed with IFN-β1a set at a final concentration of 10 LU/mL independently for each assay. NAb titers were calculated using the Kawade formula [30] and are shown in Figure 8.1. As is now well established, NAbs appear from approximately 6 months following the start of treatment [17, 18, 37], initially at low titers but subsequently often increasing significantly in titer, as in the case of these two patients, over the next 12 months. While NAb titers derived from the RGA are broadly consistent with those derived from the AVA, numerical differences are apparent. In fact, for patient 1 (Fig. 8.1A), RGA-derived titers are lower throughout the whole serum series; for patient 2, conversely, they are higher than the AVA titers. This outcome illustrates how

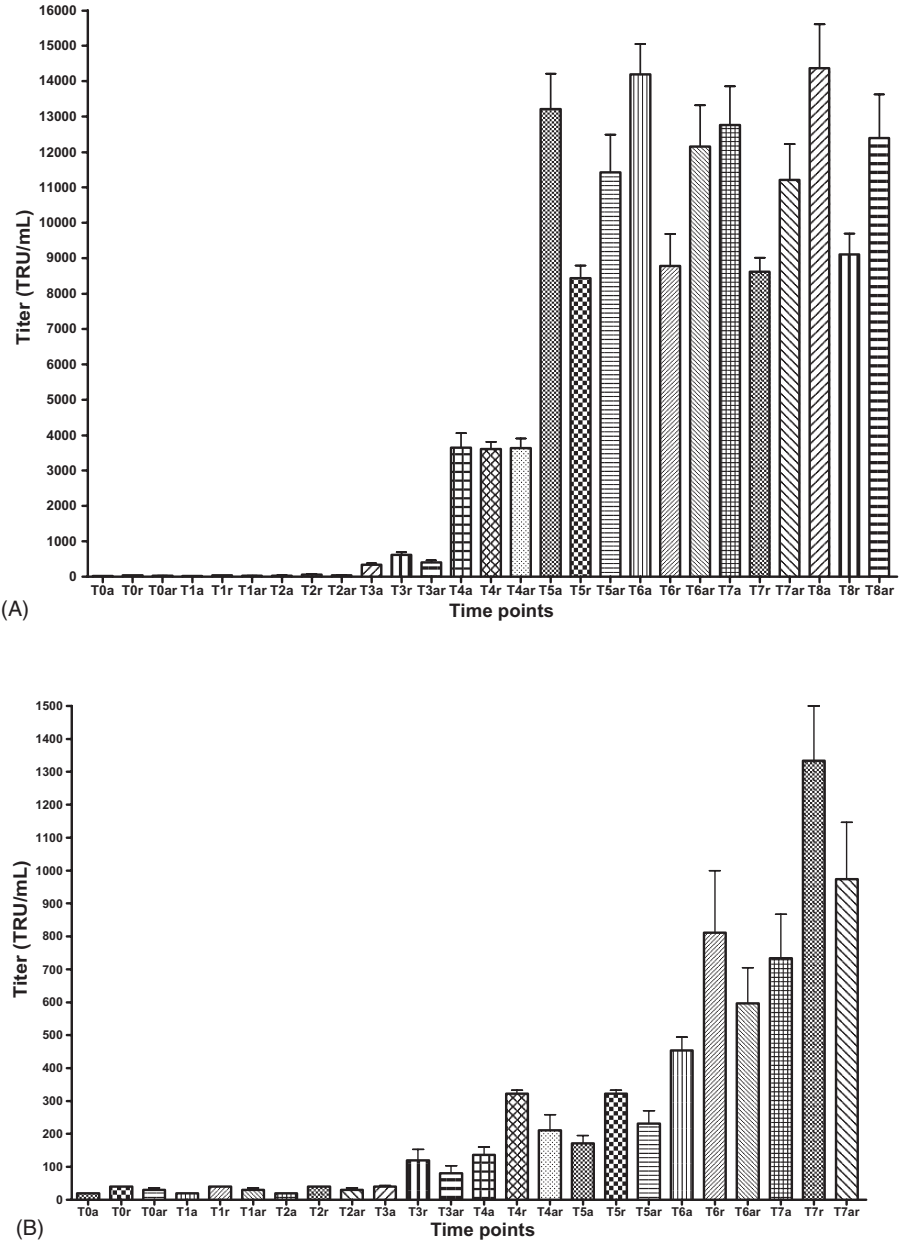

Fig. 8.1 Anti-IFN-β NAb titers in two RMMS patients, A and B, studied over a period of 2 years. Neutralizing titers were estimated in antiviral assays (a) or reporter-gene assays (r) at time points t0 (before first treatment), t1 = 3 months, t2 = 6 months, t3 = 9 months, t4 = 12 months, t5 = 15 months, t6 = 18 months, t7 = 21 months, and t8 = 24 months, and were expressed as 10-fold reduction units (TRU) per milliliter [33]. Titers are depicted as histogram bars as means of estimates from two or three separate assays. Combined titer estimates from antiviral and reporter-gene assays are shown in the "ar" histogram bars.

susceptible cell-based assays are to the heterogeneity of antibody composition and other serum components, which can have opposing effects depending on assay type. Evidently, the performance and readout of assays is influenced by factors other than their sensitivity to the added IFN-β1a.

Nevertheless, sensitivity to the molecular type of IFN-β is a consideration for NAb titer estimates. This is because of the large difference in specific activity between IFN-β1a (Avonex, Rebif) and IFN-β1b (Betaseron). The specific activity of IFN-β1a is approximately 10-fold higher than that of IFN-β1b [10–12]. While the NAbs from patients treated with either IFN-β1a or IFN-β1b are cross-reactive and neutralize both products [38–40], NAb titers are consistently lower in assays in which the IFN-β used is IFN-β1b rather than IFN-β1a [26, 41]. On a molar basis, more antibody is needed to neutralize IFN-β1b since, because of its low specific activity, its molar concentration is far higher than that of IFN-β1a to generate 10 LU/mL. Each manufacturer will prefer to use their own IFN-β product in the assay, and this undoubtedly will influence the ability of assays to detect NAbs, particularly those with low titers. This situation may worsen with the ever-growing number of subsequent-entry or biosimilar IFN-β products that are now being marketed in developing countries. The specific activity of these products may vary considerably from that of innovator IFN-β products (i.e., Avonex, Rebif for IFN-β1a; Betaseron for IFN-β1b).

Since there have been large reported differences in NAb titers, a reference assay that uses the same reagents, format, and conditions has been considered as a means of producing harmonious results from different laboratories. Among regulators of marketing authorization of biotherapeutics, there has also been growing concern that not enough was being done to address this "problem," which could have safety and efficacy implications. For instance, the European Medicines Agency (EMEA) was so concerned that it instigated a collaborative study among the three major manufacturers [Biogen-Idec, Berlex (Schering AG), and Merck Serono] of innovator IFN-β products to test a common panel of sera from RRMS patients in a common "reference assay" based on NAb inhibition of IFN-β-induced MxA protein in the A549 human lung carcinoma cell line [25]. The collaborative study was commissioned by the Biotech Working Party of the EMEA and organized by the Cytokine and Growth Factor Section of Biotherapeutics, National Institute for Biological Standards and Control (NIBSC; UK): assay data were evaluated by Informatics, NIBSC. Serum samples with titers previously determined to be negative, high, medium, and low that had been obtained by the manufacturers from IFN-β-treated RRMS patients in clinical trials were subdivided and blind-coded at NIBSC and distributed to the laboratories of the three manufacturers. Applying the constant-IFN method (described earlier in this section) using first human fibroblast-derived IFN-β and second their own innovator IFN-β product as added IFN-β, the assays were performed with A549 cells. A secondary MxA protein ELISA with an anti-MxA mAb provided by Biogen-Idec was utilized to quantify induced MxA. The assay was designed so that the results could be

reported as the reduction of 10 LU/mL to 1 LU/mL, following the procedure of Kawade [30, 31]. When fibroblast-derived IFN-β was used, there was essentially 100% concordance of NAb titers obtained among the three laboratories, with a range of 3–10% coefficient of variation. However, titers obtained against IFN-β1b as the added IFN-β were often lower than those obtained against the two IFN-β1a products. The full results of this study are currently unpublished. Although the MxA methodology designed for use in this study was considered to be acceptable, the lack of availability of the anti-MxA mAb due to patent restrictions has until recently severely restricted its general use.

8.4. NEUTRALIZING-ANTIBODY TITER AND CLINICAL RELEVANCE

The development and neutralizing capacity of anti-IFN-β NAbs in MS patients is highly variable. Prevalences of anti-IFN-β NAbs in patients treated with Avonex, Rebif (IFN-β1a), and Betaseron (IFN-β1b) were reported in 2004 to be 2–6%, 12–28%, and 28–47%, respectively [39]. However, some variation of these numbers is expected among the now numerous studies that have been conducted, largely due to the variety of methods used to measure NAbs [42]. A more recent report [15] indicated an overall prevalence of 32% in 1115 patients treated with one of the three available IFN-β products: 13% in Avonex-treated patients, 30–39% in Rebif-treated patients, and 43% in Betaseron-treated patients. The higher occurrence of NAbs in patients treated with either Rebif or Betaseron may partly be attributed to their subcutaneous administration [6]. Interestingly, the immunogenicity of Rebif has been found to be reduced by reformulating the product without human serum albumin [43]. Anti-IFN-β NAbs in individual patients have been found to appear either transiently or intermittently from about 6 months post-treatment commencement, or once formed to remain throughout the treatment regime [44]. Although detection and quantification of NAbs has been fairly inconsistent for the reasons given earlier, it is evident that NAb titers vary widely among individual patients, both at set time points and in those patients followed longitudinally over extended time periods. However, it has become increasingly evident that persistent anti-IFN-β NAbs are associated with reduced pharmacodynamics in the induction of IFN-β-responsive genes and loss of bioactivity in general [36, 37, 42, 45–47]. Although there has not been total agreement, accruing evidence strongly suggests that a lack of IFN-β bioactivity due to anti-IFN-β NAbs is associated with reduced clinical responses assessed either by magnetic resonance imaging (MRI) or by increased relapses or disease progression [17, 37, 42, 48–55]. Switching patients from one IFN-β product to another one appears to yield no significant change in anti-IFN-β NAb titers [56]. Current European guidelines recommend all patients receiving IFN-β therapy should be monitored for anti-IFN-β NAbs, and that those who develop persistent titers should discontinue the treatment. However, it is difficult to

predict and is a major challenge to decide at what level of neutralizing capacity anti-IFN-β NAbs have serious clinical impact. The cutoff NAb titer at which treatment resistance occurs is probably variable among patients, so it is not possible to give a definitive figure for this. Finally, investigation into the formation of immune complexes of anti-IFN-β NAbs with therapeutic IFN-β, which may cocirculate with free anti-IFN-β NAbs, is warranted since their influence on NAb titer determinations in bioassays and clinical responses could be significant.

8.5. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic, chronic inflammatory autoimmune disorder that leads to progressive joint damage and affects about 1% of the Caucasian populations [57–59, 60]. Although RA can affect a patient at any age, the peak incidence is in the third through fifth decade of life, and women are affected more frequently than men. The onset of the disease can be highly variable; however, most patients experience an insidious progression of joint stiffness, pain, and swelling. Over time most patients develop a characteristic pattern of joint involvement characterized by the symmetric involvement of the small joints of the hands and feet. The chronic proliferative and inflammatory nature of the disease results in erosion and destruction of joint cartilage, bone, and supporting structures, resulting in typical joint deformities, morbidity, and even early mortality.

8.6. TNF-α-INHIBITOR TREATMENT OF RHEUMATOID ARTHRITIS

A number of therapies are available for RA treatment. The mainstays of RA therapy are disease-modifying antirheumatic drugs (DMARDs), so called because these agents were thought to actually reduce joint destruction, thereby maintaining and improving function [61]. The DMARD most often employed is methotrexate (MTX) [62, 63]. However, investigation into the underlying causes of RA has led to the recognition that the proinflammatory cytokine TNF-α plays a key role in the pathogenesis of chronic inflammation [57, 58, 64, 65]. Thus, it has been possible, with advancements in biotechnology, to prepare TNF-α inhibitors (TIs) and use these in RA therapies that have rational physiologic disease-modifying potential [61, 65–67]. Biologic RA therapeutics currently on the market include two anti-TNF-α mAbs: infliximab (Remicade, Centocor, Inc., Malvern, PA) and adalimumab (Humira, Abbott Laboratories, North Chicago, IL) [66]. Infliximab, which was approved by the US Food and Drug Administration (FDA) in 1999 for use in RA, is a mouse–human chimera that joins the variable regions of a mouse antibody that is specific for TNF-α to the constant region of human IgG1(κ). Adalimumab,

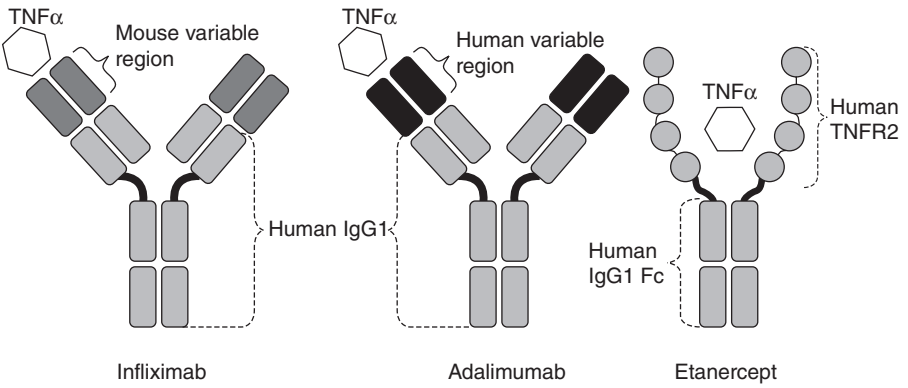

Fig. 8.2 Diagram of TNF-α inhibitors. Infliximab is a mouse–human chimeric monoclonal antibody that joins the variable regions of a mouse antibody to the constant regions of human IgG1. Adalimumab is a fully human IgG1 monoclonal antibody. Etanercept is a dimeric fusion protein that joins TNF-R2 ectodomains to the Fc domains of human IgG1.

which was approved by the FDA in 2003 for use in RA, is a fully human IgG1(κ) anti-TNF-α antibody (Fig. 8.2). Both strongly neutralize the biological activity of TNF-α. In addition, etanercept (Enbrel, Amgen, Thousand Oaks, CA, and Wyeth Pharmaceuticals, Philadelphia, PA) is a dimeric fusion protein that joins the extracellular domain of human p75 TNF-receptor (or TNF-R2) to the Fc domain of human IgG1 (Fig. 8.2); it acts as a soluble TNF-R2 that binds TNF-α strongly, effectively abrogating TNF-α-mediated activities signaled through cell-membrane TNF-Rs. Newer products are also coming into the market: golimumab (Simponi, Centocor and Schering-Plough), another fully human anti-TNF-α mAb, has recently been approved in both the United States and the European Union for use in RA; certolizumab pegol (Cimzia, UCB Inc., Smyrna, GA, USA), a pegylated humanized anti-TNF-α Fab′ fragment, has been given marketing authorization in the United States for use in RA and Crohn's disease.

Infliximab, adalimumab, and etanercept have all proved beneficial therapeutically in RA, especially in the early stages of the disease [61, 65–68]. However, efficacy is significantly improved by combination with MTX [69–71]. With all three TIs, trials have demonstrated that upward of 50–70% of enrolled patients had at least a 20% response and upward of 40% of patients had a 50% improvement as measured by the American College of Rheumatology (ACR) criteria. While each of the cohorts studied in these trials was slightly different, no significant side effects were apparent in the combination arms above and beyond what was seen in the MTX-only arms of the studies, indicating a good safety profile and strong benefit-to-risk ratio when put into perspective with the relative severity of the disease. Thus, the "gold standard" for severe active RA has become the use of a TI plus MTX; the three innovator

products have, in general, shown similar efficacy potential when used in concert with MTX [66]. A downside to this combination therapy is that high doses of TI are required to maintain pharmacological activity, making a full course of treatment rather expensive. For example, infliximab is given by intravenous infusion at a recommended dose of 3 mg/kg over approximately 2 hours. With a half-life in RA subjects of 8–10 days, the standard infusion schedule (induction phase) for RA is administration at 0, 2, and 6 weeks and every 8 weeks thereafter (maintenance phase) [66]. In contrast, adalimumab is administered subcutaneously every other week at a dose of 40 mg and has a circulating half-life of 10–20 days [66, 72]. The recommended dose of etanercept for adult RA patients is 50 mg per week given as one subcutaneous injection; its half-life is approximately 4 days [66, 73].

Despite the increased efficacy of TI in combination with MTX, a significant proportion of patients fail to respond to treatment [69–71] or lose clinical response over time [67]. One explanation for the latter is the formation of anti-TI antibodies [74–83]. Since infliximab, adalimumab, and etanercept each contain structural elements that are potentially recognized as foreign by patients' immune systems, it is not unexpected that administration of these TIs will elicit immune responses, especially those leading to the development of antibodies against these proteins. It is presumed that infliximab, which contains both murine and human protein components, can potentially elicit the production of both antiallotypic and anti-idiotypic antibodies, while adalimumab, which is a fully human mAb, is expected to lead to only anti-idiotypic antibodies in patients. As with IFN-β and other biotherapeutic proteins, such antibodies can be non-neutralizing or neutralizing. So far, antibodies against TIs have been detected and measured by a variety of immunoassay methods, which do not discriminate between non-neutralizing and neutralizing antibodies. Analysis of sera from patients in clinical trials of TI has produced widely varying anti-TI antibody prevalences. For infliximab, prevalence of anti-infliximab antibodies in RA appears to vary from 7% to 53%; it is lower with high-dose regimes and also is reduced when infliximab is given with MTX [74–77, 81–83]. The immunogenicity of adalimumab, a fully human mAb, should be much lower than the chimeric infliximab, but while some clinical studies have suggested a low prevalence, ~6% (reduced to 1% when combined with MTX) [71, 77, 82], newer more sensitive immunoassays are predicting much higher prevalence [80, 84]. One report has found a prevalence of anti-adalimumab antibodies as high as 87% [78]. For etanercept, the prevalence of anti-etanercept antibodies has ranged from 0% to 6% [79, 85].

While detrimental effects of non-neutralizing antibodies cannot be ruled out, it is much more likely that neutralizing antibodies (NAbs) will severely compromise continued therapy with a TI. For instance, the presence of NAbs to infliximab may be inferred since the detection of anti-infliximab antibodies has been associated with the development of infusion reactions, decreased levels of infliximab 4 weeks postinfusion, and a reduction in therapeutic efficacy over time [77, 81, 83]. Similar loss of responsiveness to adalimumab has

also been reported for anti-adalimumab antibody-positive RA patients [78, 81, 84], but no study has yet shown a clear association of anti-etanercept antibodies with lower clinical response [76, 79, 85]. Regulatory authorities have responded to the "threat" posed by NAbs by requiring screening of patients for anti-TI immune responses during clinical trials [86]. However, compared with the detection and measurement of anti-IFN-β antibodies in MS patients, detection and measurement of anti-TI antibodies in RA patients has proved far more challenging, particularly for detecting NAbs in cell-based assays. Since high levels of TI may remain in patients' circulation at the times of blood withdrawals [66, 72, 73, 77], the neutralizing activity of antibodies can be obscured as they are probably completely bound to the inhibitor. A major hurdle to overcome is therefore the separation of any anti-TI antibodies from immune complexes of antibody plus inhibitor prior to application to cell-based assays. In practice, such separation is proving difficult to achieve. This notwithstanding, the following section will review the current cell-based approaches for measuring NAbs against TI.

8.7. CELL-BASED ASSAY APPROACHES FOR DETECTING NEUTRALIZING ANTIBODIES TO TNF-α INHIBITORS

Tumor necrosis factor alpha (TNF-α), the prototypic member of a large family of related proinflammatory and immunoregulatory cytokines, collectively termed TNF superfamily ligands, has been extensively characterized biologically. Its activities include induction of cytotoxicity or apoptosis in many tumor cell lines, stimulation of cytokine and chemokine production, and enhancement of cellular adhesion molecule expression [87–89]. Traditionally, TNF-α has been quantified by cell-based assays (bioassays) in which a cytotoxic response is manifested. Early development of bioassays in the 1970s employed the L-929 cell line, which despite its murine origin succumbed to human TNF-α-mediated cytotoxicity [90]. It was soon established that cytotoxicity was significantly augmented by combining TNF-α with metabolic inhibitors, such as the transcription inhibitor actinomycin D (AMD) [90–92]. While the L-929 cell line plus AMD format has been the mainstay for measuring TNF-α potency, other murine and human cell lines have subsequently been found usable for this purpose. These include the murine LM and WEHI 164 clone 13 and the human HeLa, U-937, and KYM-1 lines [92]. The human rhabdomyosarcoma cell line KYM-1 and its cloned sublines have proved extremely useful for estimating potencies of TNF-α preparations [93, 94]. All of these "cytotoxicity" assays may be calibrated and validated with the WHO IS for TNF-α, 88/786 (obtainable from NIBSC).

While the cytotoxicity assays are readily adapted to quantify neutralizing anti-TNF-α antibodies, they are not easily calibrated since reference NAb standards are not available. Nevertheless, the neutralizing capacity of infliximab, adalimumab, other anti-TNF-α mAbs, and other TI such as the soluble

TNF-R2/Fc fusion protein, etanercept, can be gauged by this means. The usual practice is to pre-incubate a fixed concentration of TNF-α, one which on its own induces approximately 90–95% cell death, with serial dilutions of a TI for 2 hours. The residual un-neutralized TNF-α is then determined by addition of the mixtures to TNF-α-susceptible cells, such as L-929, WEHI 164 clone 13, or KYM-1 [95]. From the dose–response data, the TI concentration at which TNF-α-mediated cytotoxicity is reduced to an arbitrarily chosen, but fixed, level is ascertained. Results may be expressed as TI concentration, for example, nanograms per milliliter (ng/mL), or as a titer that is proportional to the dilution of the original preparation or product. "Units" of inhibitor activity may be calculated by employing a formula similar to that of Kawade and others [30–33] for anti-IFNs (see Section 8.3).

To detect and quantify NAbs against TI requires an additional step, in what has been termed an indirect NAb assay [96]. First, serial dilutions of the NAb-containing serum sample are pre-incubated for 2 hours with a fixed concentration of TI. In the absence of NAb, the TI concentration should be adjusted so that it effectively neutralizes added TNF-α. In the second step a fixed concentration of TNF-α is added to the mixtures of serial NAb dilutions and TI, and incubation continues for another 2 hours before adding to cells. In theory, the NAbs against the TI should neutralize its binding to TNF-α and thus "restore" TNF-α-mediated cytotoxicity in the bioassay. Dose–responses should show the NAb dilution at which cytotoxicity is 50% of the maximum. This dilution thus gives a measure of the neutralizing activity of the NAbs. Generally, such bioassays can be set up successfully with positive control antibodies, for example, hyperimmune animal polyclonal anti-TI sera, which contain no TI. For example, sheep polyclonal anti-etanercept serum strongly neutralizes etanercept binding to TNF-α, and "releases" its cytotoxic activity in WEHI clone 13 cells (Fig. 8.3). However, appropriate serum controls are required since the result will be dependent on interactions between the serum matrix, the TNF-α-susceptible cell line, TNF-α, and the TI [96]. For patient sera, the high doses of TI administered during treatment schedules and relatively long inhibitor half-lives have created severe difficulties of obtaining serum samples not containing any inhibitor. Thus, serum samples containing TI have proved extremely difficult to assay for NAbs since these almost certainly will be bound to some extent in immune complexes with the inhibitor. If in excess, the circulating TI will strongly interfere in the pre-incubation stages of the assay and confound its outcome. This is illustrated when sheep anti-etanercept serum is spiked with increasing concentrations of etanercept, these leading to considerable proportionate decreases in its neutralizing titer (Fig. 8.3). To detect NAbs in sera from patients may therefore require their separation from immune complexes and the elimination of residual TI. A procedure involving an acid dissociation protocol applied to immune complexes of anti-IL-1R mAb with anti-anti-IL-1R mAb has been successfully developed [97]. This and similar methods applied to immunoassays [98–100] may prove useful for detecting NAbs against TI in cell-based assays, but are yet to be reported. An additional,

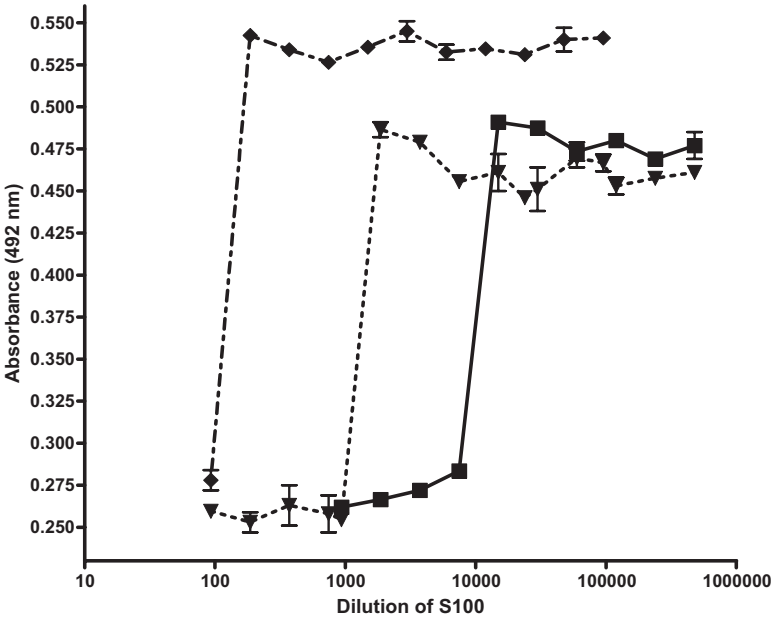

Fig. 8.3 Neutralization of etanercept by sheep S100 polyclonal anti-etanercept serum. Serial dilutions of S100 were preincubated with 500 ng etanercept per milliliter and then with 400 pg TNF-α/mL before addition to WEHI164 clone 13 monolayers. After incubation at 37°C overnight, cytotoxicity was assessed by MTS uptake and subsequent spectrophotometric measurement of its formazan product [92]. To study the effects of exogenous etanercept, S100 was spiked with etanercept at 5 and 50 μg/mL prior to the pre-incubation step. Absorbances were plotted against the reciprocal of S100 dilutions and NAb titers estimated as the S100 dilutions at which TNF-α-mediated cytotoxicity was 50% of maximum. (■) S100, no added etanercept, titer = 10,000; (△) S100 plus 5 μg etanercept/mL, titer = 1000; (◆) S100 plus 50 μg etanercept/mL, titer = 100.

potential complication when the TI is a soluble receptor, such as etanercept, is that anti-TI antibodies may bind to the cell-membrane-bound receptor of the assay cell line. For anti-etanercept antibodies, which could bind cell-membrane-bound TNF-R2, this may not greatly influence the outcome as TNF-α-induced cytotoxicity is mediated via the TNF-R1 [87–89].

In principle, alternative bioactivity assays for TNF-α could also be used to detect NAbs against TI. For instance, TNF-α is a strong inducer of the chemokines interleukin-8 (IL-8) and growth-related oncogene alpha (GRO-α) and strongly upregulates intercellular cellular adhesion molecule-1 (ICAM-1) expression in endothelial cells and some tumor cell lines, all of which are readily quantifiable [101, 102]. Bioassays based on these TNF-α-mediated activities could be adapted to measure TI, but they would suffer from the same drawbacks as cytotoxicity assays for detection and measurement of NAbs against TI.

Recently, a novel approach using cells not to measure TNF-α activity, but rather as presenters of noncleavable TNF-α, has shown promise for detecting NAbs against TI [103]. For assay development, the human T-cell line Jurkat was transfected with noncleavable TNF-α, leading to high-level expression of TNF-α at the cell surface. Binding of biotinylated TI was measured using flow cytometry or Meso Scale Discovery (MSD) electrochemiluminescence (ECL) technology. NAbs against two TI, adalimumab and a fully humanized anti-TNF-α mAb, GNExTNFvF, were generated in cynomolgus monkeys. In the MSD assay, the Jurkat cells are immobilized onto high-bind carbon-coated 96-well plates. Following incubation of biotinylated TI with sera plus or minus anti-TI antibodies, biotinylated TI bound to TNF-α was detected using streptavidin–ruthenium, which gives an ECL readout. NAbs block the binding of biotinylated TI to the Jurkat cell membrane bound TNF-α. The MSD assay proved superior in performance to the flow cytometry assay, and it was highly tolerant to added TI, suggesting that it could be used to analyze human sera from patients treated with infliximab or adalimumab, even if residual levels of these were present. In the absence of validated bioactivity assays for detecting NAbs against TI, the MSD assay, or similar, may represent the only viable alternative for this purpose. However, it remains to be determined whether it is necessary to use transfected cells or whether noncell approaches, for example, using immobilized recombinant TNF-α, would suffice to yield equivalent results.

REFERENCES

1. Mitchell, G. (1993). Update on multiple sclerosis therapy. *Contemp Clin Neurol*, *77*, 231-249.
2. Blumenthal, S. (2006). Multiple sclerosis. *Radiol Technol*, *77*, 309–321.
3. Barnett, M.H., Parratt, J.D., Pollard, J.D., Prineas, J.W. (2009). MS: is it one disease? *Int MS J*, *16*, 57–65.
4. Clerico, M., Contessa, G., Durelli, L. (2007). Interferon-beta1a for the treatment of multiple sclerosis. *Expert Opin Biol Ther*, *7*, 535–542.
5. Lam, S., Wang, S., Gottesman, M. (2008). Interferon-β1b for the treatment of multiple sclerosis. *Expert Opin Drug Metab Toxicol*, *4*, 1111–1117.
6. Goodin, D.S. (2005). Treatment of multiple sclerosis with human beta interferon. *Int MS J*, *12*, 96–108.
7. Hosoi, K., Utsumi, J., Kitagawa, T., Shimizu, H., Kobayashi, S. (1988). Structural characterization of fibroblast human interferon-beta. *J Interferon Res*, *8*, 375–384.
8. Kagawa, Y., Takasaki, S., Utsumi, J., Hosoi, K., Shimizu, H., et al. (1988). Comparative study of the asparagines-linked sugar chains of natural human interferon-β1 and recombinant human interferon-β1 produced by three different mammalian cells. *J Biol Chem*, *263*, 17508–17515.

9. Utsumi, J., Mizuno, Y., Hosoi, K., Okano, K., Sawada, R., et al. (1989). Characterization of four different mammalian-cell derived recombinant human interferon-β1s. *Eur J Biochem*, *181*, 545–553.
10. Runkel, L., Meier, M., Pepinsky, R.B., Karpusas, M., Whitty, A., et al. (1998). Structural and functional differences between glycosylated and non-glycosylated forms of human interferon beta (IFN-beta). *Pharm Res*, *15*, 641–649.
11. Meager, A., Gaines Das, R. (2005). Biological standardization of human interferon beta: establishment of a replacement World Health Organization international biological standard for human glycosylated interferon beta. *J Immunol Methods*, *306*, 1–15.
12. Karpusas, M., Whitty, A., Runkel, L., Hochman, P. (1998). The structure of human interferon-beta: implications for activity. *Cell Mol Life Sci*, *54*, 1203–1216.
13. The IFNβ Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group. (1996). Neutralizing antibodies during the treatment of multiple sclerosis with interferon beta-1b: experience during the first three years. *Neurology*, *47*, 889–894.
14. Ross, C., Klemmesen, K.M., Svenson, M., Sorensen, P.S., Koch-Henriksen, N., et al. (2000). Immunogenicity of interferon-β in multiple sclerosis patients: influence of preparation, dosage, dose frequency, and route of administration. *Ann Neurol*, *48*, 706–712.
15. Sominanda, A., Rot, U., Suoniemi, M., Deisenhammer, F., Hillert, J., Fogdell-Hahn, A. (2007). Interferon beta preparations for the treatment of multiple sclerosis patients differ in neutralizing antibody seroprevalence and immunogenicity. *Mult Scler*, *13*, 208–214.
16. Pachner, A.R. (2003). An improved ELISA for screening for neutralizing anti-IFN-beta antibodies in MS patients. *Neurology*, *61*, 1444–1446.
17. Perini, P., Calabrese, M., Biasi, G., Gallo, P. (2004). The clinical impact of interferon beta antibodies in relapsing remitting MS. *J Neurol*, *251*, 305–309.
18. Grossberg, S. E., Kawade, Y. (2006). The development and measurement of antibodies to interferon. In *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, pp. 375–399.
19. Gneiss, C., Brugger, M., Fogdell-Hahn, A., Rudzki, D., Hillert, J., et al. (2008). Comparative study of four different assays for the detection of binding antibodies against interferon-beta. *Mult Scler*, *14*, 830–836.
20. Aarskog, N.K., Marøy, T., Myhr, K.M., Vedeler, C.A. (2009). Antibodies against interferon-beta in multiple sclerosis. *J Neuroimmunol*, *212*, 148–150.
21. Gneiss, C., Reindl, M., Berger, T., Lutterotti, A., Ehling, R., et al. (2004). Epitope specificity of neutralizing antibodies against IFN-beta. *J Interferon Cytokine Res*, *24*, 283–290.
22. Meager, A. (2002). Biological assays for interferons. *J Immunol Methods*, *261*, 21–36.
23. Meager, A. (2006). Measurement of interferon activities. In *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, pp. 339–373.
24. Meager, A. (2003). Assays for antiviral activity. *Methods Mol Biol*, *249*, 121–134.

25. Pungor, E. Jr., Files, J.G., Gabe, J.D., Do, L.T., Foley, W.P., et al. (1998). A novel bioassay for the determination of neutralizing antibodies to IFN-β1. *J Interferon Cytokine Res*, *18*, 1025–1030.
26. Lallemand, C., Meritet, J.F., Erickson, R., Grossberg, S.E., Roullet, E., et al. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
27. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., et al. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321*, 19–31.
28. Moore, M., Meager, A., Wadhwa, M., Burns, C. (2009). Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type 1 interferon-inducible 6–16 mRNA. *J Pharm Biomed Anal*, *49*, 534–539.
29. WHO Report of the Expert Committee on Biological Standardization. (1996). *WHO Tech Rep Ser*, *858*, 11.
30. Kawade, Y. (1980). An analysis of neutralization reaction of interferon by antibody: a proposal on the expression of neutralization titer. *J Interferon Res*, *1*, 61–70.
31. Kawade, Y. (1986). Quantitation of neutralization of interferon by antibodies. *Methods Enzymol*, *119*, 558–573.
32. Grossberg, S.E., Kawade, Y., Kohase, M., Yokoyama, H., Finter, N. (2001). The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. *J Interferon Cytokine Res*, *21*, 729–742.
33. Grossberg, S.E., Kawade, Y., Kohase, M., Klein, J.P. (2001). The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. *J Interferon Cytokine Res*, *21*, 743–755.
34. Grossberg, S.E., Kawade, Y., Grossberg, L.D. (2009). The neutralization of interferons by antibody. III. The constant antibody bioassay, a highly sensitive quantitative detector of low antibody levels. *J Interferon Cytokine Res*, *29*, 93–104.
35. Lam, R., Farrell, R., Aziz, T., Gibbs, E., Giovannoni, G., Grossberg, S., Oger, J. (2008). Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFNbeta in multiple sclerosis patients. *J Immunol Methods*, *336*, 113–118.
36 McKay, F., Schibeci, S., Heard, R., Stewart, G., Booth, D. (2006). Analysis of neutralizing antibodies to therapeutic interferon-beta in multiple sclerosis patients: a comparison of three methods in a large Australian cohort. *J Immunol Methods*, *310*, 20–29.
37. Farrell, R.A., Giovannoni, G. (2007). Measuring and management of anti-interferon beta antibodies in subjects with multiple sclerosis. *Mult Scler*, *13*, 567–577.
38. Antonelli, G., Simeoni, E., Bagnato, F., Pozzilli, C., Turriziano, O., et al. (1999). Further study on the specificity and incidence of neutralizing antibodies to interferon (IFN) in relapsing remitting multiple sclerosis patients treated with IFN-beta-1a or IFN-beta-1b. *J Neurol Sci*, *168*, 131–136.

39. Bertolotto, A., Deisenhammer, F., Gallo, P., Sölberg Sørensen, P. (2004). Immunogenicity of interferon beta: differences among products. *J Neurol*, *251* (Suppl. 2), II15–II24.
40. Khan, O.A., Dhib-Jalbut, S.S. (1998). Neutralizing antibodies to interferon beta-1a and interferon beta-1b in MS patients are cross-reactive. *Neurology*, *51*, 1698–1702.
41. Bertolotto, A., Malucchi, S., Milano, E., Castello, A., Capobianco, M., Mutani, R. (2000). Interferon beta neutralizing antibodies in multiple sclerosis: neutralizing activity and cross-reactivity with three different preparations. *Immunopharmacology*, *48*, 95–100.
42. Deisenhammer, F. (2009). Neutralizing antibodies to interferon-beta and other immunological treatments for multiple sclerosis: prevalence and impact on outcomes. *CNS Drugs*, *23*, 379–396.
43. Giovannoni, G., Barbarash, O., Casset-Semanaz, F., King, J., Metz, L., et al. (2009). Safety and immunogenicity of a new formulation of interferon beta-1a (Rebif New Formulation) in a phase IIIb study in patients with relapsing multiple sclerosis: 96-week results. *Mult Scler*, *15*, 219–228.
44. Sorensen, P.S., Koch-Henriksen, N., Ross, C., Clemmesen, K.M., Bentzen, K., and the Danish Multiple Sclerosis Study Group. (2005). Appearance and disappearance of neutralizing antibodies during interferon-beta therapy. *Neurology*, *65*, 33–39.
45. Bertolotto, A., Gilli, F., Sala, A., Capobianco, M., Malucchi, S., et al. (2003). Persistent neutralizing antibodies abolish the interferon beta bioavailability in MS patients. *Neurology*, *60*, 634–639.
46. Scagnolari, C., Duda, P., Bagnato, F., De Vito, G., Alberelli, A., et al. (2007). Pharmacodynamics of interferon beta in multiple sclerosis patients with or without serum neutralizing antibodies. *J Neurol*, *254*, 597–604.
47. Pachner, A., Narayan, K., Pak, E. (2006). Multiplex analysis of expression of three IFNβ genes in antibody-positive MS patients. *Neurology*, *66*, 444–446.
48. Pachner, A.R. (2007). Neutralizing anti-IFNβ antibodies: clarity and confusion. *Mult Scler*, *13*, 1081–1082.
49. Koch-Henriksen, N., Sorensen, P.S., Bendtzen, K., Flachs, E.M. (2009). The clinical effect of neutralizing antibodies against interferon-beta is independent of the type of interferon-beta used for patients with relapsing-remitting multiple sclerosis. *Mult Scler*, *15*, 601–605.
50. Bertolotto, A. (2007). Implications of neutralizing antibodies on therapeutic efficacy. *J Neurol Sci*, *277* (Suppl. 1), S29-S32.
51. Hartnung, H.P., Polman, C., Bertolotto, A., Deisenhammer F., Giovannoni, G., et al. (2007). Neutralising antibodies to interferon beta in multiple sclerosis: expert panel report. *J Neurol*, *254*, 827–837.
52. Petkau, J., White, R.A., Ebers, G.C., Reder, A.T., Sibley, W.A., et al. (2004). Longitudinal analyses of the effects of neutralizing antibodies on interferon beta-1b in relapsing-remitting multiple sclerosis. *Mult Scler*, *10*, 126–138.
53. Namaka, M., Politt-Smith, M., Gupta, A., Klowak, M., Vasconcelos, M., et al. (2006). The clinical importance of neutralizing antibodies in relapsing-remitting multiple sclerosis. *Curr Med Res Opin*, *22*, 223–239.

54. van der Voort, L.F., Visser, A., Knol, D.L., Oudejans, C.B., Polman, C.H., Killestein, J. (2009). Lack of interferon-beta bioactivity is associated with the occurrence of relapses in multiple sclerosis. *Eur J Neurol*, *16*, 1049–1052.

55. Pachner, A.R., Cadavid, D., Wolansky, L., Skurnick, J. (2009). Effect of anti-IFNβ antibodies on MRI lesions of MS patients in the BECOME study. *Neurology*, *73*, 1485–1492.

56. Gneiss, C., Koudouovoh-Tripp, P.M., Roplele, S., Gotwald, T., Ehling, R., et al. (2009). Influence of interferon-beta therapy switching on neutralizing antibody titres: results from the Austrian Switch Study (ASS). *Mult Scler*, *13*, Nov 13 [Epub ahead of print].

57. Choy, E.H., Panayi, G.S. (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med*, *344*, 907–916.

58. Feldmann, M., Maini, R.N. (2001). Anti-TNFα therapy of rheumatoid arthritis: what have we learned? *Annu Rev Immunol*, *19*, 163–196.

59. Tobón, G.J., Youinou, P., Saraux, A. (2009). The environment, geo-epidemiology, and autoimmune disease: rheumatoid arthritis. *Autoimmun Rev*, Nov 27 [Epub ahead of print].

60. Lee, D.M., Weinblatt, M.E. (2002). Rheumatoid arthritis. *Lancet*, *358*, 903–911.

61. Feely, M.G., Erickson, A., O'Dell, J.R. (2009). Therapeutic options for rheumatoid arthritis. *Expert Opin Pharmacother*, *10*, 2095–2106.

62. Kremer, J.M. (1999). Methotrexate and leflunomide: biochemical basis for combination therapy in the treatment of rheumatoid arthritis. *Semin Arthritis Rheum*, *29*, 14–26.

63. Kremer, J.M., Alarcón, G.S., Lightfoot, R.W. Jr., Wilkens, R.F., Furst, D.E., et al. (1994). Methotrexate for rheumatoid arthritis. Suggested guidelines for monitoring liver toxicity. American College of Rheumatology. *Arthritis Rheum*, *37*, 316–328.

64. Arend, W.P., Dayer, M. (1995). Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. *Arthritis Rheum*, *38*, 151–160.

65 Tracey, D., Klareskog, L., Sasso, E.H., Salfield, J.G., Tak, P.P. (2008). Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther*, *117*, 244–279.

66. Kukar, M., Petryna, O., Efthimiou, P. (2009). Biological targets in the treatment of rheumatoid arthritis: a comprehensive review of current and in-development biological disease modifying anti-rheumatic drugs. *Biologics*, *3*, 443–457.

67 Criscione, L.G., St Clair, E.W. (2002). Tumor necrosis factor-alpha antagonists for the treatment of rheumatic diseases. *Curr Opin Rheumatol*, *14*, 204–211.

68 Breedveld, F.C., Emery, P., Keystone, E., Patel, K., Furst, D.E., et al. (2004). Infliximab in active early rheumatoid arthritis. *Ann Rheum Dis*, *63*, 149–155.

69. Lipsky, P.E., van der Heijde, D.M., St Clair, E.W., Furst, D.E., Breedvald, F.C., et al. (2000). Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. *N Engl J Med*, *343*, 1594–1602.

70. Weinblatt, M.E., Kremer, J.M., Bankhurst A.D., Bulpitt, J.J., Fleischmann, R.M., et al. (1999). A trial of etanercept, a recombinant tumor necrosis factor receptor:

Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *N Engl J Med*, *340*, 253–259.

71. Weinblatt, M.E., Keystone, E.C., Furst, D.E., Moreland, L.W., Weisman, M.H., et al. (2003). Adalimumab, a fully human anti-tumor necrosis factor monoclonal antibody, for the treatment of rheumatoid arthritis in patients taking concomitant methotrexate: the ARMADA trial. *Arthritis Rheum*, *48*, 35–45.
72. Adalimumab package insert. [cited 01/2008]. Available at http://www.rxabbott.com/pdf/humira.pdf
73. Etanercept package insert. [cited 10/2007]. Available at http://www.enbrel.com/pdf/enbrel_pi.pdf
74. Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., et al. (1998). Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor α monoclonal antibody combined with low-dose methotrexate in rheumatoid arthritis. *Arthritis Rheum*, *41*, 1552–1563.
75. Wagner, C.L., Schantz, A., Barnathan, E., Olson, A., Mascelli, M.A., et al. (2003). Consequences of immunogenicity to the therapeutic monoclonal antibodies ReoPro and Remicade. *Dev Biol (Basel)*, *112*, 37–53.
76. Anderson, P.J. (2005). Tumor necrosis factor inhibitors: clinical implications of their different immunogenicity profiles. *Semin Arthritis Rheum*, *34*, 19–22.
77. Bendtzen, K., Geborek, P., Svenson M., Larrson L., Kapetanovic, M.C., Saxne, T. (2006). Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor α inhibitor infliximab. *Arthritis Rheum*, *54*, 3782–3789.
78. Bender, N.K., Helig, C.E., Dröll, B., Wohlgemuth, Amdruster, F.P., Helig, B. (2007). Immunogenicity, efficacy and adverse events of adalimumab in RA patients. *Rheumatol Int*, *27*, 269–274.
79. Dore, R.K., Mathews, S., Schechtman, J., Surbeck, W., Mandel, D., et al. (2007). The immunogenicity, safety and efficacy of etanercept liquid administered once weekly in patients with rheumatoid arthritis. *Clin Exp Rheumatol*, *25*, 40–46.
80. Aarden, L., Ruuls, S.R., Wolbink, G. (2008). Immunogenicity of anti-tumor necrosis factor antibodies—toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20*, 431–435.
81. Radstake, T.R., Svenson, M., Eijsbouts, A.M., van den Hoogen, F.H., Enevold, C., van Riel, P.L., Bendtzen, K. (2009). Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. *Ann Rheum Dis*, *68*, 1739–1745.
82. Aikawa, N.E., de Carvalho, J.E., Almeida Silva, C.A., Bonfá, E. (2009). Immunogenicity of anti-TNF-α agents in autoimmune diseases. *Clin Rev Allerg Immunol*, June 30 [Epub ahead of print].
83. Harauoi, B., Cameron, L., Ouellet, M., White, B. (2006). Anti-infliximab antibodies in patients with rheumatoid arthritis require higher doses of infliximab to achieve or maintain a clinical response. *J Rheumatol*, *33*, 31–36.
84. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Staple, S., Lems, W.F., et al. (2007). Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis*, *66*, 921–926.

85. Keystone, E.C., Schiff, M.H., Kremer, J.M., et al. (2004). Once weekly administration of 50 mg etanercept in patients with active rheumatoid arthritis: results of a multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum*, *50*, 353–363.
86. Shankar, G., Shores, E., Wagner, C., Mire-Sluis, A. (2006). Scientific and regulatory considerations on the immunogenicity of biologics. *Trends Biotechnol*, *24*, 274–280.
87. Jäätelä, M. (1991). Biologic activities and mechanisms of action of tumour necrosis factor-α/cachectin. *Lab Invest*, *64*, 724–742.
88. Bayaert, R., Fiers, W. (1998). *Tumour necrosis factor and lymphotoxin*. In Cytokines. Mire-Sluis A, Thorpe R, eds. Academic Press, San Diego, pp. 335–360.
89. Collette, Y., Gilles, A., Pontarotti, P., Olive, D. (2003). A co-evolution perspective of the TNFSF and TNFRSF families in the immune system. *Trends Immunol*, *24*, 387–394.
90. Matthews, N., Neale, M.L. (1987). Cytotoxicity assays for tumour necrosis factor and lymphotoxin. In *Lymphokines and Interferons—A Practical Approach*. Clemens MJ, Morris A, Gearing AJH, eds. IRL Press, *Oxford*, pp. 221–225.
91. Meager, A., Leung, H., Woolley, J. (1987). Assays for tumour necrosis factor and related cytokines. *J Immunol Methods*, *116*, 1–17.
92. Meager, A. (2003). Assays for cytotoxicity. *Methods Mol Biol*, *249*, 135–151.
93. Meager, A. (1991). A cytotoxicity assay for tumour necrosis factor using a human rhabdomyosarcoma cell line. *J Immunol Methods*, *144*, 141–143.
94. Meager, A. (1999). A tumour necrosis factor-alpha (TNF-α) sensitive adherent KYM-1D4 derived cell line: use in TNF-α cytotoxicity assays in the presence of actinomycin D. *J Immunol Methods*, *227*, 197–198.
95. Meager, A. (2006). Measurement of cytokines by bioassays: theory and application. *Methods*, *38*, 237–252.
96. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., et al. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
97. Lofgren, J.A., Wala, I., Koren, E., Swanson, S.J., Jing, S. (2006). Detection of neutralizing anti-therapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J Immunol Methods*, *308*, 101–108.
98. Patton, A., Mullenix, M.C., Swanson S.J., Koren, E. (2005). An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J Immunol Methods*, *304*, 189–195.
99. Bourdage, J.S., Cook, C.A., Farrington, D.L., Chain, J.S., Konrad, R.J. (2007). An affinity capture elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. *J Immunol Methods*, *327*, 10–17.
100. Smith, H.W., Butterfield, A., Sun, D. (2007). Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid phase extraction with acid-dissociation (SPEAD) sample treatment prior to ELISA. *Regul Toxicol Pharmacol*, *49*, 230–237.
101. Goebeler, M., Yoshimura, T., Toksky, A., Ritter, U., Bröcker, E.B., Gillitzer, R. (1997). The chemokine repertoire of human dermal microvascular endothelial

cells and its regulation by inflammatory cytokines. *J Invest Dermatol*, *108*, 445–451.

102. Meager, A. (1996). Bioimmunoassays for proinflammatory cytokines involving cytokine-induced cellular adhesion molecule expression in human glioblastoma cell lines. *J Immunol Methods*, *190*, 235–244.
103. Loyet, K.M., Deng, R., Liang, W.-C., Wu, Y., Lowman, H.B., DeForge, L.E. (2009). Technology comparisons for anti-therapeutic antibody and neutralizing antibody assays in the context of an anti-TNF pharmacokinetic study. *J Immunol Methods*, *345*, 17–28.
104. LaFleur, D.W., Nardelli, B., Tsareva, T., Mather, D., et al. (2001). Interferon-kappa, a novel type I interferon expressed in human keratinocytes. *J Biol Chem*, *276*, 39765–39771.
105. Seo, Y-J., Kim, G-H., Kwak, H-J., Nam, J-S., Lee, H.J., et al. (2009). Validation of a HeLa *Mx2*/Luc reporter cell line for the quantification of human type I interferons. *Pharmacology*, *84*, 135–144.

9

DETECTION OF NEUTRALIZING ANTIBODIES AGAINST INTERFERON BETA BY REAL-TIME RT-PCR

Francesca Gilli and Antonio Bertolotto

9.1. INTRODUCTION

Clinical studies have demonstrated that recombinant interferon beta-1b (IFN-β1b) and recombinant interferon beta-1a (IFN-β1a) are effective treatments for patients with relapsing–remitting multiple sclerosis (RRMS). These treatments may fail, however, because of noncompliance with therapy, intolerable adverse effects, nonresponsive disease, or the development of anti-IFN-β neutralizing antibodies (NAbs).

Neutralizing antibodies are formed in up to a third of patients [1] and are capable of reducing or abrogating the normal biological [2–8] and treatment effects of IFNs [9–19]. One mechanism by which NAbs affect IFN-β therapy is by their binding directly to the epitope of the IFN-β molecule that binds to the IFN-β receptor, neutralizing the effect of IFN-β on target cells [20].

As the therapeutic effects of IFN-β are delayed after initiation of therapy, there is probably some lag time before the clinical effects of NAbs become evident. Therefore, it has been recommended that NAbs be monitored periodically, perhaps even before there is clinical evidence of loss of efficacy, to help assess the degree of clinical response to IFN-β [21]. Based on these recommendations, several different assay systems have been developed to measure

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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NAbs, chief among which are, partly for historical reasons, antiviral assays [22]. Indeed, anti-IFN-β NAbs can be measured using a bioassay based on the antiviral activity of the cytokine, known as the cytopathic effect assay (CPE) [23]. Specifically, this assay is based on the observation that IFN-β inhibits the cytopathic effect of viruses on cultured cells. In the presence of serum containing NAbs, IFN-β is neutralized, resulting in the death of the infected cells. The extent of the virus cytopathic effect, caused by dilutions of samples from patients, allows a determination of the degree of neutralization and, thus, a NAb titration.

This latter bioassay still represents the "gold standard" for NAb measurements, as stated by the World Health Organization (WHO) and by the European Federation of Neurological Societies (EFNS) [21], even if it is laborious and time-consuming and frequently displays poor reproducibility, mostly due to the inherent variability of challenge virus infection and replication. Hence, to improve reproducibility, alternative bioassays have been developed that do not require virus challenge.

One recently favored for quantifying NAbs against IFN-β is a protocol involving the induction of the antiviral protein myxovirus resistant protein A (MxA) by IFN-β in a cell bioassay followed by the quantitative measurement of MxA by enzyme-linked immunosorbent assay (ELISA) [24–26]. Specifically, this assay, termed MxA protein induction assay (MPA), measures the inhibition by serum of the IFN-β induction of the cellular MxA protein. Clearly, this assay offers several advantages over the traditional CPE assay; for example, it has no need of viruses, is easier to standardize, has the possibility of being automated, and has safer methodology. On these grounds, the European Medicines Agency (EMEA) in 2008 recommended the MPA as the reference assay for anti-IFN-β NAb testing instead of the CPE assay [27]. Nevertheless, although it has greater simplicity and reliability, the MPA is as time-consuming as a CPE assay, and the ELISA (used for measuring MxA protein) requires specific anti-human MxA antibodies that are not commercially available. To overcome these technical problems, some new, modified MPA bioassays have been introduced: further developments include bioassays based on the induction of IFN-responsive genes, as well as the use of the highly reproducible and sensitive technology of real-time reverse transcription polymerase chain reaction (real-time RT-PCR) [28–31].

The first modified bioassay method was published in 2007 [28]. The latter is a protocol analyzing, once again, the induction of the antiviral protein MxA by IFN-β, but involving the use of real-time RT-PCR technology to quantitate MxA gene expression instead of protein levels [28]. Afterward, alternative bioassay formats have been investigated, trying to simplify the reliability test procedure; these approaches differ in terms of mRNAs measured, reference genes, quantitation approaches, and extraction–amplification protocols.

More recently, a new approach to the detection of NAbs to IFNs involves bioassays based on the IFN-induced expression of a reporter gene. These assays use cells stably transfected with a plasmid containing, for example, the

MxA promoter linked to an enzyme such as firefly luciferase. While such assays have been used for IFN potency determinations [32, 33], and only more recently for measuring NAbs to IFNs [34], they do not require the use of the real-time RT-PCR technology.

9.2. AN INTRODUCTION TO REAL-TIME RT-PCR

In molecular biology, real-time RT-PCR is a laboratory technique based on polymerase chain reaction (PCR), which is used to amplify and simultaneously quantify a targeted mRNA molecule, retrotranscripted in cDNA. It enables both detection and quantification (as an absolute number of copies or as a relative amount when normalized to cDNA input or additional normalizing genes) of one or more specific sequences in a mRNA sample.

The procedure follows the general principle of PCR; its key feature is that the amplified cDNA is detected as the reaction progresses in *real time*, a new approach compared with standard PCR, where the product of the reaction is detected at its end.

The discovery of the real-time RT-PCR technique as it is used today was made possible by two important findings. First, the Taq-polymerase has, apart from its polymerase activity, a 5′-3′ exonuclease activity [35]. Second, dual-labeled fluorogenic oligonucleotide probes have been created that emit a fluorescent signal only upon cleavage, based on the principle of fluorescence resonance energy transfer [36]. In the TaqMan assay, which was the first real-time RT-PCR assay developed, these two principles are combined. In this system a probe, the so-called TaqMan® probe, is designed to anneal to the target sequence between the classical forward and reverse primers (Fig. 9.1A). The probe is dually labeled, with a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at one end and a quencher dye (e.g., 6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3′ end. Importantly, in its intact form, the fluorescence emission of the reporter dye is absorbed by the quencher dye (Fig. 9.1A). It does this by the use of fluorescence (or Förster) resonance energy transfer (FRET), which is the inhibition of one dye caused by another without emission of a proton. Once the TaqMan probe has bound to its specific piece of the template after denaturation (high temperature) and the reaction cools, the primers anneal to the cDNA. Taq-polymerase then adds nucleotides and cleaves the TaqMan probe from the template (Fig. 9.1B). This separates the quencher from the reporter, and allows the reporter to emit its energy (Fig. 9.1C).

The amount of fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation. The light emitted from the dye in the excited state is focused onto a charge-coupled device (CCD), which is a light-sensitive integrated circuit that converts light into a digital signal. The computer then shows the digital signal on a graph display, showing PCR cycles

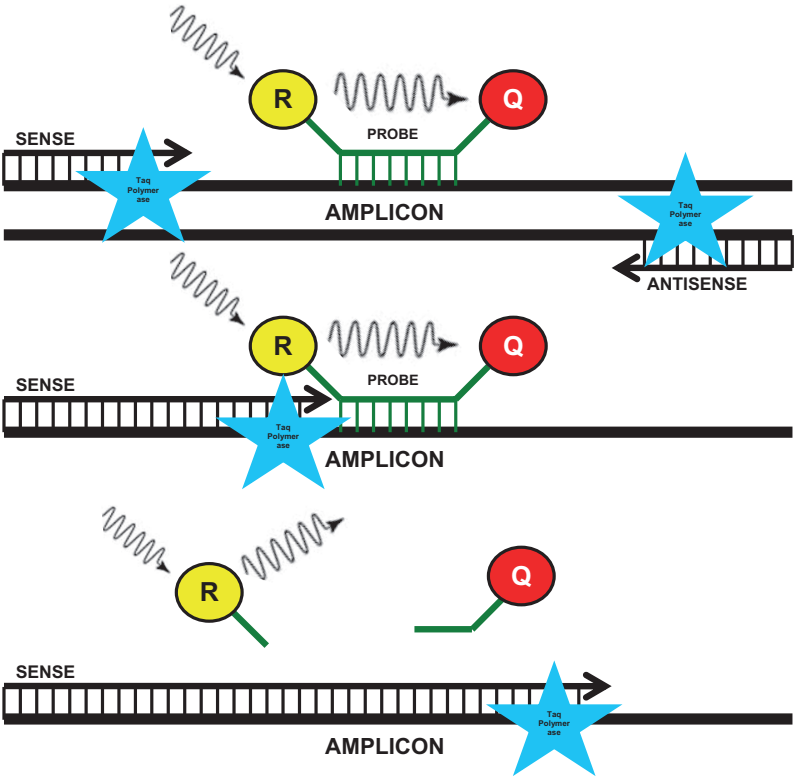

Fig. 9.1 TaqMan probe chemistry mechanism. TaqMan probes consist of a reporter (R), that is, a fluorophore covalently attached to the 5′-end of the oligonucleotide probe, and a quencher (Q) at the 3′-end. Several different fluorophores [e.g., 6-carboxyfluorescein, (FAM), or tetrachlorofluorescin (TET)] and quenchers [e.g., tetramethylrhodamine (TAMRA) or dihydrocyclopyrroloindole tripeptide *minor groove binder* (MGB)] are available. The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source via fluorescence resonance energy transfer (FRET). (A) As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. (B) TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5′ to 3′ exonuclease activity of the polymerase degrades the probe that has annealed to the template. (C) Degradation of the probe releases the fluorophore (R) from it and breaks the close proximity to the quencher (Q), thus relieving the quenching effect and allowing fluorescence of the fluorophore. Hence, fluorescence detected in the real-time RT-PCR thermal cycler is directly proportional to the fluorophore released and the amount of cDNA template present in the PCR. (See color insert.)

on the x-axis and a logarithmic indication of fluorescence intensity on the y-axis.

Real-time RT-PCR is a kinetic approach in which you look at the reaction in the early stages while it is still linear. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline, and the point at which the fluorescence crosses the threshold is called the cycle threshold, Ct. So the higher the initial amount of the sample, the sooner accumulated product is detected in the PCR process as a significant increase in fluorescence, and the lower the Ct value. Ct values are very reproducible in replicates because the threshold is picked to be in the exponential phase of the PCR, where there is a linear relation between the logarithm of the change in fluorescence and cycle number and where the reaction components are not limiting. The quantity of mRNA theoretically doubles every cycle during the exponential phase and relative amounts of mRNA can be calculated; for example, a sample whose Ct is 2 cycles earlier than another's has $2^2 = 4$ times more template.

In the real-time RT-PCR method, specific errors can be introduced due to minor differences in starting amount of RNA or differences in efficiency of cDNA synthesis and PCR amplification. Therefore, a reliable real-time RT-PCR requires correction for these experimental variations. At present this is most often performed by normalization to a housekeeping gene, that is, dividing the measured amount of mRNA from the gene of interest by the amount of mRNA from the housekeeping gene, measured in the same sample. This normalization permits accurate comparison of expression of the gene of interest between different samples, provided that the expression of the housekeeping gene used in the normalization is very similar across all the samples. Choosing a reference gene fulfilling this criterion is therefore highly important, and often challenging, because only very few genes show equal levels of expression across a range of different conditions or tissues. Many recent articles have discussed the problem of housekeeping genes, and it turns out that the housekeeping genes most commonly used are subject to variation in numerous experimental conditions, bringing into question the reliability of the results obtained. In particular, the use of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and of actin (ACT) has been severely criticized [37–39]. Ribosomal RNA (18S rRNA) may be a more valuable alternative, because its expression is less likely to vary under conditions that change the levels of mRNA expression. Drawbacks of using rRNA, however, are that it cannot be used for quantification of samples when oligo $d(T)_{16}$ is used for cDNA synthesis [40].

Two strategies can be employed to quantify the results obtained by real-time RT-PCR; the standard curve method (i.e., a number of copies of a specific RNA per sample) and the comparative threshold method (i.e., sample A has twice as much mRNA of a specific gene as sample B).

In the standard curve method, a standard curve is first constructed from an RNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for mRNA targets of unknown concentrations.

The second quantitation approach is termed the comparative Ct method. This involves comparing the Ct values of the samples of interest with a control or calibrator such as an untreated sample or mRNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene. The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$; ΔCt_{sample} is the Ct value for any sample normalized to the endogenous housekeeping gene, and $\Delta Ct_{reference}$ is the Ct value for the calibrator, also normalized to the endogenous housekeeping gene.

An important advantage of using TaqMan probes is the possibility of measuring multiple mRNA species in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified and permits quantification of mRNAs in single-tube, homogeneous assays.

9.3. EXPERIMENTAL: NEUTRALIZING ANTIBODIES MEASURED BY REAL-TIME RT-PCR ASSAYS

9.3.1. IFN-β Treatment

By far, the majority of real-time RT-PCR bioassay protocols involve the induction of IFN-responsive genes by IFN-β in a human lung carcinoma A549 cell line obtained from either the European Collection of Animal Cell Cultures (ECACC) or the American Type Culture Collection (ATCC). Cells are commonly cultured in flat 96-well tissue culture plates in cell growth medium and incubated overnight to form a confluent monolayer.

The real-time RT-PCR bioassay is based on the measurement of the inhibition by serum of the IFN-β induction of specifically induced genes (e.g., MxA): in the presence of serum containing NAbs, IFN-β is neutralized, resulting in a low amount of IFN-β-induced mRNAs. The extent of the specific gene induction, caused by dilutions of samples from patients, allows a determination of the degree of neutralization and, thus, a NAb titration.

Cells are exposed to IFN-β, diluted in culture media at 20 laboratory units per milliliter (LU/mL; the LU is defined as the smallest amount of IFN-β that produces the endpoint of the assay) and an equal amount of test sera in a serial twofold dilution (1:2.5 up to 1:5120), resulting in a final IFN-β concentration of 10 LU/mL. This specific IFN-β dilution allows calculation of the NAb titer as the dilution of serum that reduces the amount of LU/mL of IFN-β by 90% (1 LU/mL = 50% MxA induction) [41–44].

9.3.2. Isolation of Total RNA

After stimulation and incubation of cells, samples are removed by aspiration and cells are lysed for RNA extraction. RNA isolation, indeed, is required to have complementary DNA (cDNA), necessary for gene expression analysis.

Total RNA is extracted from both unstimulated cells (representing the background control) and IFN-β-stimulated A549 cells, employing either manual (e.g., RNeasy spin-column technology, from Qiagen, or NucleoSpin technology, from Macherey Nagel) or semiautomatic (e.g., the ABI PRISM 6100 Nucleic Acid PrepStation, Applied Biosystems) extraction systems. Clearly, these two different extraction systems (i.e., manual RNA extraction vs. semiautomated RNA extraction) were compared, showing that with both systems RNA is efficiently recovered from the A549 cells [28].

The cDNA is synthesized *in vitro* from a mRNA template using reverse transcriptase. The resulting cDNA is single-stranded. This process is called reverse transcription (RT) or first-strand cDNA synthesis. The purpose of converting mRNA to cDNA is mainly for analysis of the template mRNA because DNA is more stable than RNA. Once mRNA is converted to cDNA, the cDNA can be used for real-time RT-PCR, as a probe for expression analysis of the mRNA sequence.

Retrotranscription of the RNA can be primed by a gene-specific primer, random hexamers, or oligo $d(T)_{16}$ to produce first-strand cDNA. The choice of RT primers is a consideration that can affect the design and outcome of an experiment. Although random hexamers will preferentially prime ribosomal RNA (unless the amount of reverse transcriptase is increased), they will prime any type of RNA or DNA, including partially degraded mRNA and genomic DNA. Oligo $d(T)_{16}$, on the other hand, offers more specific priming from the poly$(A)^+$ mRNA tail or from randomly dispersed poly(A)-rich regions, giving greater assurance that the mRNA is intact. However, oligo $d(T)_{16}$ cannot be used to prime 18S rRNA, eliminating its use as an endogenous control. Nevertheless, this might be an important limitation for the real-time RT-PCR bioassay, as several studies have suggested that 18S rRNA is a more reliable endogenous control for gene expression studies than commonly used housekeeping genes, such as GAPDH or ACT [37–39].

To date, both retrotranscription methods have been used in real-time RT-PCR bioassay for NAbs, showing similar results with both random hexamers and oligo $d(T)_{16}$, when using the correct PCR format as well as the right housekeeping gene for normalization [28–31].

9.3.3. Quantitative PCR

The cDNA is then used as a template for the real-time RT-PCR analysis based on the 5′ nuclease assay described in Section 9.2. In this context, real-time quantitative RT-PCR is done to measure IFN-β-induced gene products: either

the conventionally used IFN-β-induced MxA gene, which is specifically induced by IFN-β [28, 29, 31], or an alternative IFN-inducible gene 6–16 [30], which has recently been shown to encode a 34-kDa glycosylated protein G1P3 localized in mitochondria [45] and is transcriptionally upregulated in type I IFN–treated human cell lines.

Transcriptional expression of the IFN-β-induced genes is normalized with the endogenous control of choice to avoid differences due to possible RNA degradation or contamination or to different reverse transcription efficiency. Quantitative data of the IFN-inducible gene 6–16 expression were normalized to the expression levels of the GAPDH gene, performing single-plex PCR, that is, amplifying control and target gene in separate reactions [30]. The specific choice of GAPDH as housekeeping gene was favored by the use of oligo $d(T)_{16}$ for the retrotranscription strategy. Obviously, it would be possible to change the endogenous control, also changing the strategy of reverse transcription.

On the other hand, transcriptional expression of the MxA gene was normalized using different reference genes such as GAPDH [28, 29, 31], large ribosomal protein (RPLPO) [31], human transcription factor IID (TBP) [31], and 18S rRNA [28, 29]. To evaluate the validity of normalization of MxA expression, the reliability of all those control genes was statistically assessed. All reference genes passed the test and can thus be safely used to normalize MxA gene expression.

Notably, the reference gene 18S rRNA was particularly useful for multiplexing (i.e., allowing detection of target and reference genes in the same well), as this gene is more abundantly expressed than the target gene MxA [28, 29]. Indeed, in multiplex PCR it is important to ensure that the gene expression level of the endogenous control is greater than that of the target gene. By understanding the relative abundance of target and reference species, it is possible to control the competition between the reactions. Once the relative abundance of the competing species is known, preventing domination by the abundant species becomes a simple matter of limiting the primers. By limiting the primer concentration of the most abundant gene, you will limit the amount of this product generated, allowing more reagents to be available for the less abundant genes.

The response of targets is usually expressed in terms of relative expression considering the difference in threshold cycles (Ct) for target and reference (ΔCt), after background gene expression from control wells (unstimulated cells) is subtracted. Targets are considered detectable with Ct values higher than 35.

When using constitutively expressed housekeeping genes, proving the stable expression of the selected gene is required, to achieve normalization with the same level of confidence. The ΔCt method was thus selected to avoid variability among wells and the need to run endless plasmid standard curves on every 96-well plate. The accuracy of comparing genes in this way depends on primer sets having equal amplification efficiency, which can be determined via the slope of a standard curve.

9.3.4. Definition of NAb Units

Controls of each plate include untreated cells, to check possible IFN-β expression independent of the IFN-β stimulation, and a simultaneous IFN-β titration, to measure directly the antigen activity employed in the test. Sigmoidal dose–response curves are generated from the IFN-β-induced target expression values, determining the 50% endpoint (i.e., EC_{50}) and statistical evaluations (Fig. 9.2A).

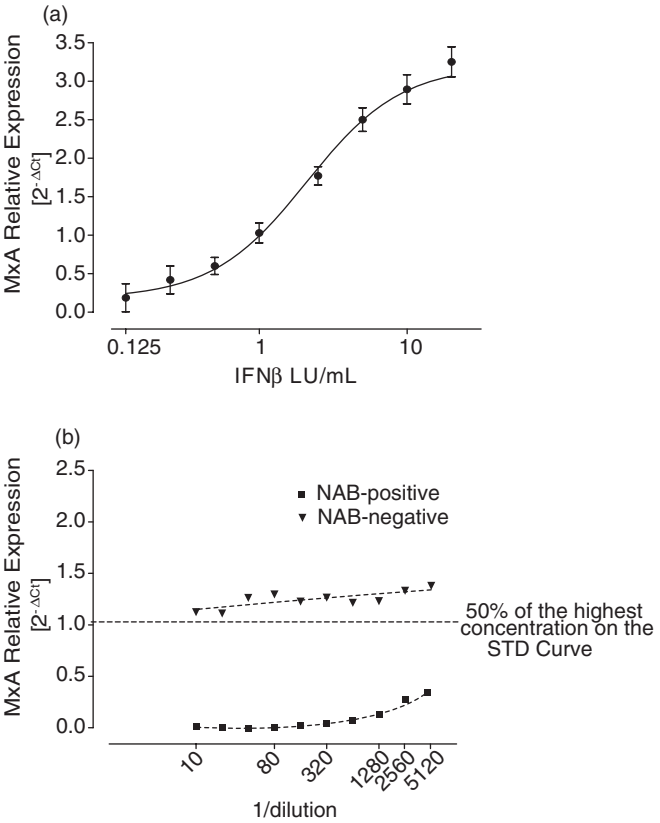

Fig. 9.2 Determination of NAb titer. (A) Standard curve of MxA induction by human NIH IFN-β reference standard used to determine EC_{50} and concentration (LU/mL) of the challenge dose. (B) MxA induction by IFN-β in the presence of serial dilutions (from 1:10 up to 1:5120) of two human sera evaluated as NAb-positive and NAb-negative. In reporting the results of NAb tests detected with the real-time RT-PCR bioassay, the EC_{50} (concentration of IFN-β inducing half-maximal MxA induction) was determined from the standard curve and was assigned a concentration of 1 LU/mL. A sample with all MxA induction ($2^{-\Delta\Delta Ct}$) values of less than 50% of the highest concentration on the standard curve was considered definitely NAb-negative. Otherwise, the sample was titrated and neutralizing activity was expressed in TRU/mL.

The Kawade approach [41, 42], based on the mathematical model of antibody–antigen interaction developed by Kawade and recommended by WHO [23], is commonly used for the computation and reporting of anti-IFN-β NAb results. In its current form it allows calculation of NAb titer from the dilution of serum that reduces 10 LU/mL of IFN-β activity to 1 LU/mL. A refinement to the calculation has enabled the titer of antibody that reduces a fixed concentration of IFN-β (may be more or less than 10 LU/mL) to 1 LU/mL to be calculated from a Kawade formula as 10-fold reduction units per milliliter (TRU/mL) [43, 44]. In reporting the results of NAb tests detected with the real-time RT-PCR bioassay, the final endpoint is 50% in the linear portion of the dose–response curve, which corresponds to stimulation with 1 LU/mL.

Before proceeding with sample titration, negative samples are commonly screened out. Specifically, screening samples are diluted 1:10, incubated with 20 LU/mL IFN-β, and tested for IFN-β-induced gene expression. A sample with a $2^{-\Delta\Delta Ct}$ value of less than 50% of the highest concentration on the standard curve is considered NAb negative (Fig. 9.2B). Otherwise, the sample is titrated and neutralizing activity is expressed in TRU/mL, as just described.

Based on the observation that IFN-β1b binds approximately 3 times more NAbs than IFN-β1a, sera are mostly tested for NAbs against the type of IFN-β for individual patients to avoid false negative test results [46]. However, it has been suggested to exploit the increase in specificity of NAbs against IFN-β1a in order to improve the clinical sensitivity of a NAb assay. On this ground several groups have decided not to test sera against the IFN-β preparation taken from the patients, but to use only IFN-β1a for the NAb determination.

In the literature, cross-reactivity of NAbs against the two different preparations of IFN-β (i.e., IFN-β1a and IFN-β1b) has been reported [46–48]. In agreement, the amount of neutralizing units (i.e., NAbs) against IFN-β1b is normally lower than the amount of neutralizing units against IFN-β1a in the same patient. Lower NAb results against IFN-β1b have been explained by the presence of denaturation and/or the formation of aggregates among molecules of IFN-β1b [46] due to the absence of glycosylation [49]. Particularly, it has been explained that each aggregate may bind only one IFN-β receptor but several NAb molecules. As gene induction by IFN-β depends on the number of IFN-α/β receptors (IFNARs) bound by IFN-β, several molecules of IFN-β1b do not exert any biological activity in the presence of aggregates. Hence, based on the observation that IFN-β1b binds approximately 3 times more NAbs than IFN-β1a, sera are mostly tested for NAbs against the type of IFN-β for individual patients, to avoid false negative test results [46]. However, it has been suggested to exploit the increase in specificity of NAbs against IFN-β1a in order to improve the clinical sensitivity of a NAb assay. On this ground several groups have decided to use the IFN-β1a Avonex (Biogen Idec, Cambridge, Mass.) for the NAb determination and not to test sera against the IFN-β preparation taken by the patients.

9.3.5. Dose-Dependent Gene Expression Induced by IFN-β in A549 Cells

Lysates of uninduced A549 cells do not contain detectable IFN-β-induced gene expression. This was assessed by quantifying the gene expression of MxA in untreated cells [28]: in wells with untreated cells, MxA Ct was always lower than 35 (meaning no significant amplification), while housekeeping genes (i.e., 18S rRNA and GAPDH) were always detectable

Stimulation of A549 cells with different dilutions of IFN-β normally shows a dose-dependent induction of IFN-β-induced mRNAs from 0.125 LU/mL (lower limit of quantification, LLOQ) to greater than 30 LU/mL (upper limit of quantification, ULOQ) of IFN-β (Fig. 9.2A). The lowest quantity of IFN-β inducing a signal distinguishable from the absence of the cytokine (blank value) is 0.0625 LU/mL (limit of detection, LOD). For analysis purposes the linear part of the sigmoidal response curve is usually employed, defined as between a lower and an upper limit: for the IFN-β dose–response curve, the response is nearly linear in the range of 0.5–20 LU/mL of IFN-β.

To provide a calibration of the different preparations of IFN-β, dose–response curves obtained with either IFN-β1a or IFN-β1b were compared with curves obtained after stimulation with human recombinant IFN Reference Standard NIH-Gb23–902–531. All values calculated for the NIH reference IFN-β over the range 0.125–30 LU/mL were equal to the expected concentrations within the SD of the assay for both IFN-β1a and IFN-β1b (±7.8%) [28].

9.4. TECHNICAL ISSUES

At present there is high variability among laboratories with regard to the methods used for measuring NAbs [50]. Most published data on anti-IFN-β NAbs were obtained with the CPE assay, which is based on the observation that IFN-β inhibits the cytopathic effect of viruses on cultured cells; more recently, the neutralization assay based on the inhibition of IFN-β protein expression (i.e., MxA protein) has been introduced. This new assay has greater simplicity and reliability because it does not involve the use of viruses and avoids concerns about virus titration and the stability of stock viruses. Further developments include a bioassay based on a new technical procedure that has increased reliability and reproducibility compared with either of the two conventional bioassays for NAb measurement. As stated previously, this procedure involves the use of real-time RT-PCR technology to quantitate IFN-β-induced gene induction instead of protein levels. Compared with the traditional ELISA method (used for measuring IFN-β-induced MxA), real-time RT-PCR technology is more expensive; however, it is much faster, less susceptible to variability problems, and utilizes readily available reagents. It also should be taken into consideration that mRNA levels are more strongly correlated with the interaction between IFN-β and its receptor, giving a more precise measurement of the degree of inhibition of cellular effects induced by

IFN-β due to the presence of NAbs. Indeed, the protein level produced by the cell does not always reflect the mRNA level. Although this correlation generally holds true, some situations have been observed in which the gene is highly expressed but translational, rather than transcriptional, mechanisms control the amount of protein produced by the cell [51, 52].

Technically, an important advantage of measuring mRNA levels by real-time RT-PCR over the conventional NAb bioassays is the possibility of controlling for the amount of starting material. When dealing with *in vitro* cell cultures, it can be difficult to estimate cell numbers because cells will often clump together or have different morphologies, particularly when cultured as a monolayer. Moreover, cells and cultures grown in rich media can produce substantially higher cell densities. These higher densities will undoubtedly affect the response, with greater total response from higher cell densities and vice versa. Differences in cell density can lead to significant variability between laboratories, as well as between tests performed in the same laboratory. Consequently, when using a NAb assay, cell plating density must be strictly controlled. However, for both conventional bioassays (i.e., the CPE and the MPA), no internal controls for cell plating density are yet available. On the contrary, when using a real-time RT-PCR assay any bias from cell density can be easily checked and corrected thanks to normalizing genes.

Normalizing to a reference gene is a simple and popular method for internally controlling for error in real-time RT-PCR. This strategy targets RNA encoded by genes, which are collectively called housekeeping genes, and benefits from the fact that all steps required to obtain the final PCR measurement are controlled for. Reference genes can also control for different input cell plating densities or differences in cell growth or different expressivity in culture subjected to several passes. Thus, the possibility of controlling for the amount of starting material significantly improves the performance characteristics of the real-time RT-PCR bioassay with respect to both the CPE and the MPA bioassays.

To test whether higher or lower cell density significantly affects the mRNA quantification and whether this variation can be compensated by normalization of gene expression levels, A549 cells were plated at different plating densities (twofold increases). Cells were then stimulated with 20 LU/mL of IFN-β, and RNA was extracted. After retrotranscription, Ct values for MxA and GAPDH were evaluated by real-time RT-PCR, showing that Ct values varied at different input cell densities, resulting in about 1 cycle of difference between wells (e.g., cDNA concentration doubled in each well in which a double quantity of cells was plated). Otherwise, normalization with GAPDH resulted in a quantification of MxA mRNA expression, which was similar in each well and independent of the different cell densities [28].

Likewise, changes in reference gene expression were studied in relation to serum cytotoxicity, an effect which might affect the validity of any cell-based bioassay. Such slight cytotoxicity is easily checked and corrected thanks to the normalizing process of real-time RT-PCR.

Variability is also decreased in that the real-time RT-PCR can be performed using multiple dyes, and thus it is a multiplex PCR procedure. Multiplex real-time RT-PCR has both advantages and disadvantages, depending on the target genes under investigation and the efficiency of the PCR reactions to detect each gene. One of the advantages of multiplexing is that normalizing in the same well will account for variability in both pipetting accuracy and individual PCR assay variability, such as inhibitory factors. Another is the cost savings from using less master mix. A disadvantage is the need for maximum optimization of reactions. However, it is noteworthy that reagents are now commercialized as preoptimized for multiplex PCR.

As a whole, all validation results indicate that the real-time RT-PCR bioassay can be used as a substitute for both conventional NAb bioassays, because a strong correlation was found in different laboratories among NAb titers detected by the real-time RT-PCR bioassay and both the CPE assay and the MPA. However, the final analysis of variability in all three bioassays shows a higher reproducibility in the real-time RT-PCR bioassay.

9.5. PRACTICAL ISSUES

The accurate and reliable NAb titration by real-time RT-PCR bioassay requires the use of an integrated approach based on the use of several techniques: cell culture, RNA extraction, RNA retrotranscription, and real-time RT-PCR amplification. This methodology is more expensive as it combines conventional and molecular tests in order to get better results. While automation of the whole process and the use of real-time RT-PCR reduce the hands-on time in the laboratory and require significantly less optimization, it does make the bioassay more expensive.

RNA isolation is required to have cDNA necessary for gene-expression analysis and, thus, NAb titration. Two different extraction systems have been compared: manual RNA extraction and semiautomated RNA extraction. The list prices of the processing kits (i.e., costs per specimen) for the manual extraction system are generally more expensive than prices reported for the consumables used with the semiautomatic workstations. On the other hand, it is obvious that laboratory equipment for semiautomated RNA extraction (e.g., ABI 6100 Workstation) is much more expensive than equipment for manual handling, and several assays will need to be run on it to justify its purchase. The total time for runs with a comparable number of specimens is significantly greater for the manual extraction system than for the semiautomated extraction system.

When common quality assurance guidelines for molecular diagnostic are followed [53], real-time RT-PCR requires less scientific expertise for performance and analysis than both CPE and ELISA. Thus, the initial cost of a real-time RT-PCR (price of the machine plus time spent optimizing the assay)

would be offset by savings in labor, quality assurance, and materials in laboratories analyzing large numbers of samples.

The total amount of time (both the actual amount of a technologist's time spent processing each isolate and the turnaround time from specimen arrival in the laboratory to reporting of the final titration) was estimated for CPE, for MPA, and for real-time RT-PCR. These estimates assumed a common starting time subsequent to sera collection and storage. Real-time RT-PCR takes less than 3 days from start to finish and after the reaction is set up does not require any hands-on time by the technologist. CPE assay requires approximately 4 working days to perform and is much more labor intensive. In addition, regular virus titration is needed. The MPA test takes the longest time (5 working days) to perform and requires extensive hands-on work by the technologist, both for setup and for ongoing evaluation.

In addition to NAb detection assays, an MxA induction test *in vivo* is being developed to assess the response of patients to IFN-β and to assess the biological consequences of NAbs [1, 4–8]. This assay measures MxA mRNA concentrations in peripheral blood mononuclear cells of patients after injection of IFN-β, and represents an important tool supporting NAb measurement in MS patients' management. As a consequence, the same technology and reagents used in the real-time RT-PCR bioassay can also be used for the evaluation of biological activity *in vivo*.

In conclusion, each of the real-time RT-PCR bioassay formats offers important advantages when compared with the existing, commonly used assays. This new strategy can be easily used in specialized laboratories to offer reproducible quantification of NAbs in sera of IFN-β-treated MS patients, and thus it allows for a better tailoring of IFN-β therapy in every patient.

REFERENCES

1. Bertolotto, A., Malucchi, S., Sala, A., Orefice, G., Carrieri, P.B., Capobianco, M., Milano, E., Melis, F., Giordana M.T. (2002). Differential effects of three interferon betas on neutralising antibodies in patients with multiple sclerosis: a follow up study in an independent laboratory. *J Neurol Neurosurg Psychiatry*, *73*, 148–153.
2. Deisenhammer, F., Reindl, M., Harvey, J, Gasse, T., Dilitz, E., Berger, T. (1999). Bioavailability of interferon beta 1b in MS patients with and without neutralizing antibodies. *Neurology*, *52*, 1239–1243.
3. Vallittu, A.M., Halminen, M., Peltoniemi, J., Ilonen, J., Julkunen, I., Salmi, A., Erälinna, J.-P., and the Finnish Beta-Interferon Study Group. (2002). Neutralizing antibodies reduce MxA protein induction in interferon-beta 1a treated MS patients. *Neurology*, *58*, 1786–1790.
4. Bertolotto, A., Gilli, F., Sala, A., Capobianco, M., Malucchi, S., Milano, E., Melis, F., Marnetto, F., Lindberg, R.L.P., Bottero, R., Di Sapio, A., Giordana. M.T. (2003). Persistent neutralizing antibodies abolish the interferon-beta bioavailability in MS patients. *Neurology*, *60*, 634–639.

5. Bertolotto, A., Sala, A., Malucchi, S., Marnetto, F., Caldano, M., Di Sapio, A., Capobianco, M., Gilli, F. (2004). Biological activity of interferon betas in patients with multiple sclerosis is affected by treatment regimen and neutralising antibodies. *J Neurol Neurosurg Psychiatry*, *75*, 1294–1299.
6. Pachner, A.R., Narayan, K., Price, N., Hurd, M., Dail, D. (2003). MxA gene expression analysis as an interferon-beta bioactivity measurement in patients with multiple sclerosis and the identification of antibody-mediated decreased bioactivity. *Mol Diagn*, *7*, 17–25.
7. Pachner, A.R., Dail, D., Pak, E., Narayan, K. (2005). The importance of measuring IFNβ bioactivity: monitoring in MS patients and the effect of anti-IFNβ antibodies. *J Neuroimmunol*, *166*, 180–188.
8. Gilli, F., Bertolotto, A., Sala, A., Hoffmann, F., Capobianco, M., Malucchi, S., Glass, T., Kappos, L., Lindberg, R.L.P., Leppert, D. (2004). Neutralizing antibodies against IFN-beta in multiple sclerosis: antagonization of IFN-beta mediated suppression of MMPs. *Brain*, *127* (pt. 2), 259–268.
9. The IFNβ Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group. (1995). Interferon beta-1b in the treatment of multiple sclerosis: final outcome of the randomized controlled trial. *Neurology*, *45*, 1277–1285.
10. Jacobs, L.D., Cookfair, D.L., Rudick, R.A., Herndon, R.M., Richert, J.R., Salazar, A.M., Fischer, J.S., Goodkin, D.E., Granger, C.V., Simon, J.H., Alam, J.J., Bartoszak, D.M., Bourdette, D.N., Braiman, J., Brownscheidle, C.M., Coats, M.E., Cohan, S.L., Dougherty, D.S., Kinkel, R.P., Mass, M.K., Munschauer, F.E. 3rd, Priore, R.L., Pullicino, P.M., Scherokman, B.J., Whitham, R.H., et al. (1996). Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. *Ann Neurol*, *39*, 285–294.
11. Rudick, R.A., Simonian, N.A., Alam, J.A., Campion, M., Scaramucci, J.O., Jones, W., Coats, M.E., Goodkin, D.E., Weinstock-Guttman, B., Herndon, R.M., Mass, M.K., Richert, J.R., Salazar, A.M., Munschauer, F.E. 3rd, Cookfair, D.L., Simon, J.H., Jacobs, L.D. (1998). Incidence and significance of neutralizing antibodies to interferon-b 1a in multiple sclerosis. Multiple Sclerosis Collaborative Research Group (MSCRG). *Neurology*, *50*, 1266–1272.
12. The PRISMS (Prevention of Relapses and Disability by Interferon-β-1a Subcutaneously in Multiple Sclerosis) Study Group, the University of British Columbia MS/MRI Analysis Group. (2001). PRISMS-4: long-term efficacy of interferon-β-1a in relapsing MS. *Neurology*, *56*, 1628–1636.
13. Polman, C., Kappos, L., White, R., Dahlke, F., Beckmann, K., Pozzilli, C., Thompson, A., Petkau, J., Miller, D.; European Study Group in Interferon Beta-1b in Secondary Progressive MS. (2003). Neutralizing antibodies during treatment of secondary progressive multiple sclerosis with interferon beta-1b. *Neurology*, *60*, 37–43.
14. Perini, P., Calabrese, M., Biasi, G., Gallo, P. (2004). The clinical impact of interferon beta antibodies in relapsing remitting MS. *J Neurol*, *251*, 305–309.
15. Malucchi, S., Sala, A., Gilli, F., Bottero, R., di Sapio A., Capobianco, M., Bertolotto, A. (2004). Neutralizing antibodies reduce the efficacy of beta-IFN during treatment of multiple sclerosis. *Neurology*, *62*, 2031–2037.

16. Malucchi, S., Gilli, F., Caldano, M., Marnetto, F., Valentino, P., Granieri, L., Sala, A., Capobianco, M., Bertolotto, A. (2008). Predictive markers for response to interferon therapy in patients with multiple sclerosis. *Neurology*, *70*, 1119–1127.

17. Francis, G.S., Rice, G.P.A., Alsop, J.C., for the PRISMS (Prevention of Relapses and Disability by Interferon β-1a Subcutaneously in Multiple Sclerosis) Study Group. (2005). Interferon β-1a in MS: results following development of neutralizing antibodies in PRISMS. *Neurology*, *65* (1), 48–55.

18. Kappos, L., Clanet, M., Sandberg-Wollheim, M., Radue, E.W., Hartung, H.P., Hohlfeld, R., Xu, J., Bennet, D., Sandrock, A., Goelz, S., European Interferon Beta-1a IM Dose Comparison Study Group Investigators. (2005). Neutralising antibodies and efficacy of interferon beta-1a: a 4-year controlled study. *Neurology*, *65*, 40–47.

19. Sorensen, P.S., Ross, C., Clemmesen, K.M., Bendtzen, K., Frederiksen, J.L., Jensen, K., Kristensen, O., Petersen, T., Rasmussen, S., Ravnborg, M., Stenager, E., Koch-Eriksen, N., and the Danish Multiple Sclerosis Study Group. (2003). Clinical importance of neutralising antibodies against interferon beta in patients with relapsing-remitting multiple sclerosis. *Lancet*, *362*, 1184–1189.

20. Runkel, L., De Dios, C., Karpusas, M., Baker, D., Li, Z., Zafari, M., Betzenhauser, M., Muldowney, C., Miller, S., Redlich, P.N., Grossberg, S.E., Whitty, A., Hochman, P.S. (2001). Mapping of IFN-beta epitopes important for receptor binding and biologic activation: comparison of results achieved using antibody-based methods and alanine substitution mutagenesis. *J Interferon Cytokine Res*, *21* (11), 931–941.

21. Sørensena, P.S., Deisenhammer, F., Dudac, P., Hohlfeldd, R., Myhre, K.-M., Palacef, J., Polman, C., Pozzilli, C., Rossi C., for the EFNS Task Force on Anti-IFN-β Antibodies in Multiple Sclerosis. (2005). Guidelines on use of anti-IFN-β antibody measurements in multiple sclerosis: report of an EFNS Task Force on IFN-β antibodies in multiple sclerosis. *European J Neurol*, *12*, 817–827.

22. Meager, A. (2006). *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, p. 339.

23. WHO Expert Committee on Biological Standardisation. (1985). *Thirty-Fifth Report. WHO Technical Report Series*. World Health Organization, Geneva, Switzerland, p. 725.

24. Files, J.G., Gray, J.L., Do, L.T., Foley, W.P., Gabe, J.D., Nestaas, E., Pungor, E. Jr. (1998). A novel sensitive and selective bioassay for human type I interferons. *J Interferon Cytokine Res*, *18*, 1019–1024.

25. Pungor, E. Jr., Files, J.G., Gabe, J.D., Do, L.T., Foley, W.P., Gray, J.L., Nelson, J.W., Nestaas, E., Taylor, J.L., Grossberg, S.E. (1998). A novel bioassay for the determination of neutralizing antibodies to IFN-beta 1b. *J Interferon Cytokine Res*, *18*, 1025–1030.

26. McKay, F., Schibeci, S., Heard, R., Stewart, G., Booth, D. (2006). Analysis of neutralizing antibodies to therapeutic interferon-beta in multiple sclerosis patients: a comparison of three methods in a large Australasian cohort. *J Immunol Methods*, *310*, 20–29.

27. European Medicines Agency (EMEA). (2008). BWP report to the CHMP. Beta-interferons and neutralising antibodies (in multiple sclerosis). Development of a

standardised assay methodology for the determination of neutralising antibodies. London, 13 February.

28. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., Marnetto, F., Gilli, F., (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321* (1–2), 19–31.
29. Gilli, F., van Beers, M., Marnetto, F., Jiskoot, W., Bertolotto, A., Schellekens, H. (2008). Development of a bioassay for quantification of neutralising antibodies against human interferon-beta in mouse sera. *J Immunol Methods*, *336*, 119–126.
30. Moore, M., Meager, A., Wadhwa, M., Burns C. (2009). Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type 1 interferon-inducible 6–16 mRNA. *J Pharm Biomed Anal*, *49*, 534–539.
31. Aarskog, N.K., Marøy, T., Myhr, K.-M., Vedeler, C.A. (2009). Antibodies against interferon-beta in multiple sclerosis. *J Neuroimmunol*, *212*, 148–150.
32. Meager, A. (2006). In *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, p. 339.
33. Meager, A., Visvalingam, K., Dilger, P., Bryan, D., Wadhwa, M. (2005). Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine*, *31* (2), 109–118.
34. Lallemand, C., Meritet, J.-F., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., Lebon, R., Tovey, M.G. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
35. Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5′→3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA*, *88*, 7276–7280.
36. Cardullo, R.A., Agrawal, S., Flores, C., Zamecnick, P.C., Wolf, D.E. (1988). Detection of nucleic acid hybridization by non-radiative fluorescence resonance energy transfer. *Proc Natl Acad Sci USA*, *85*, 8790–8794.
37. Bustin, S.A. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol*, *25*, 169–193.
38. Schmittgen, T.D., Zakrajsek, B.A. (2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods*, *46*, 69–81.
39. Suzuki, T., Higgins, P.J., Crawford, D.R. (2000). Control selection for RNA quantitation. *Biotechniques*, *29*, 332–337.
40. Overbergh, L., Giulietti, A., Valckx, D., Decallonne, B., Bouillon, R., Mathieu C. (2003). The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech*, *14*, 33–43.
41. Kawade, Y. (1986). Quantitation of neutralization of interferon by antibody. *Methods Enzymol*, *119*, 558–573.
42. Kawade, Y., Finter, N., Grossberg, S.E. (2003). Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. *J Immunol Methods*, *278*, 127–144.

43. Grossberg, S.E., Kawade, Y., Kohase, M., Klein, J.P. (2001). The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the ten-fold reduction unit. *J Interferon Cytokine Res*, *21*, 743–755.

44. Grossberg, S.E., Kawade, Y. (2006). In *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, p. 375.

45. Tahara, E. Jr., Tahara, H., Kanno, M., Naka, K., Takeda, Y., Matsuzaki, T., Yamazaki, R., Ishihara, H., Yasai, W., Barrett, J.C., Ide, T., Tahara, E. (2005). G1P3, an interferon inducible gene 6–16, is expressed in gastric cancer and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell. *Cancer Immunol Immunother*, *54* (8), 729–740.

46. Bertolotto, A., Malucchi, S., Milano, E., Castello, A., Capobianco, M., Mutani, R. (2000). Interferon β neutralizing antibodies in multiple sclerosis: neutralizing activity and cross-reactivity with three different preparations. *Immunopharmacology*, *48*, 95–100.

47. Khan, O.A., Dhib-Jalbut, S.S. (1998). Neutralizing antibodies to interferon β-1a and β-1b in MS patients are cross-reactive. *Neurology*, *51*,1696–1702.

48. Antonelli, G., Simeoni, E., Bagnato, F., Pozzilli, C., Turriziani, O., Tesoro, R., Di Marco, P., Gasperini, C., Fieschi, C., Dianzani, F. (1999). Further study on the specificity and incidence of neutralizing antibodies to interferon (IFN) in relapsing multiple sclerosis patients treated with IFN beta-1a or IFN beta-1b. *J Neurol Sci*, *168*, 131–136.

49. Runkel, L., Meier, W., Pepinsky, R.B., Karpusas, M., Whitty, A., Kimball, K., Brickelmaier, M., Muldowney, C., Jones, W., Goelz, S.E. (1998). Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-beta (IFN-beta). *Pharmacol Res*, *15*, 641–649.

50. Hartung, H.P., Munschauer, F.E. III. (2004). Assessment and management of neutralizing antibodies in patients with multiple sclerosis. *J Neurol*, *251* (Suppl. 2), II40.

51. Greenbaum, D., Colangelo, C., Williams, K., Gerstein, M. (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol*, *4* (9), 117.

52. Mata, J., Marguerat, S., Bahler, J. (2005). Post-transcriptional control of gene expression: a genome-wide perspective. *Trends Biochem Sci*, *30* (9), 506–514.

53. Burckard, H.J. (2000). Standardization and quality control of PCR analyses. *Clin Chem Lab Med*, *38*, 87–91.

10

COMPETITIVE LIGAND-BINDING ASSAYS FOR THE DETECTION OF NEUTRALIZING ANTIBODIES

Bonnie W. Wu, George R. Gunn III, and Gopi Shankar

10.1. INTRODUCTION

A growing number of therapeutic proteins are being successfully used for the treatment of a wide range of clinical indications. Accumulated evidence from clinical trials indicates that treatment with most therapeutic proteins can elicit anti-drug antibody (ADA) responses in drug recipients [1].

Immunogenicity of protein therapeutics has become an important concern in recent years, to drug developers, clinicians, and regulatory agencies. Although often benign, ADA responses can lead to adverse clinical effects, including hypersensitivity and allergic reactions [2]. In addition, ADAs binding to the drug can lead to increased (or in some cases, decreased) clearance rates, resulting in the alteration of pharmacokinetic (PK) parameters and even loss of drug efficacy [3]. Neutralizing antibodies (NAbs), a subset of ADAs, bind at or near the active site of the drug molecule and neutralize its functional activity. In some cases, such as type I interferons, NAbs can also bind to an epitope outside of the active motif of the drug and induce conformation changes, sterically inhibiting the drug from binding to its target [4, 5]. Therefore, NAbs can limit drug efficacy and are a particular concern when they cross-react with an identical or homologous, nonredundant endogenous protein, neutralizing its biological activity. Not all NAb immune responses cause complications—antibodies to some drugs such as insulin, factor XIII, and alpha interferons were not

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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devastating and clinicians often treated the patients in the presence of ADAs [1, 6, 7]. However, some NAb responses are severe, leading to the generation of autoimmunity and clinical deficiency syndrome [8, 9]. For instance, NAbs to biologic drugs such as granulocyte–macrophage colony-stimulating factor, erythropoietin, and thrombopoietin–megakaryocyte-derived growth factor have been found to result in serious complications [1]. Therefore, assessment of the immunogenicity of protein therapeutics and its clinical consequences has become a crucial aspect for drug development and regulatory filings. A critical aspect of this assessment is characterization of the neutralizing properties of ADA responses, since it can serve as an early indicator of adverse clinical sequelae manifested during patient treatment [4].

Thorough evaluation of immunogenicity and its correlation to clinical outcome relies on the availability of robust bioanalytical methods offering adequate specificity, sufficient sensitivity, and high precision for the detection and characterization of all types of ADAs to protein therapeutics. A tiered bioanalytical strategy is commonly applied in immunogenicity testing, which includes detection and subsequent characterization of the ADA responses [10, 11]. ADA detection starts with a screening assay, which is followed by a confirmatory assay to identify ADA-positive samples. The subsequent characterization of ADAs typically includes titer measurement and the determination of NAb activity of ADA-positive samples. To ensure the detection of potentially clinically relevant ADAs, regulatory agencies typically expect ADA detection assays to possess sensitivities between 250 and 500 ng/mL to support clinical studies and 500–1000 ng/mL to support nonclinical studies [12]. In fact, recent technological advancements have enabled the immunoassay-based screening of ADAs at concentrations less than 100 ng/mL. Hence, although the sensitivity of a NAb assay should ideally also be within this range, most optimized NAb assays are commonly found to be less sensitive (usually 500–1000 ng/mL) than immunoassay-based ADA screening methods. This is due to the fact that NAb assays, cell-based methods in particular, are generally more complicated and less precise than immunoassay-based screening methods. Optimization of NAb assays, especially assay sensitivity, is critical because an absence of "detected" NAb activity might not truly confirm that the ADA is a non-neutralizing antibody; instead, the apparent lack of activity may be a consequence of insufficient assay sensitivity. Therefore, it is essential to adopt a NAb assay strategy of reasonable sensitivity, specificity, precision, and robustness to ensure meaningful and conclusive assessment of neutralizing antibody responses.

10.2. ASSAY PLATFORMS FOR DETECTION OF NEUTRALIZING ANTIBODIES

A range of technologies have been developed for the measurement of NAbs in biological fluids. These methodologies can be broadly categorized into

in vitro cell-based bioassays and competitive ligand-binding assays (CLBAs). Cell-based bioassays have traditionally been the format preferred by regulatory agencies. However, other assay platforms, especially non-cell-based CLBAs, have become attractive alternatives, largely due to the ability of CLBAs to overcome the practical and technical limitations common to cell-based NAb bioassays.

Cell-based NAb bioassays are *in vitro* functional assays utilizing cell lines that respond directly or indirectly to the drug being assessed for NAbs. A test sample's NAb activity is assessed by the sample's ability to neutralize the drug-induced cellular response in a dose-dependent manner [4]. Such cellular responses can be measured using different assay endpoints, including early biological responses (e.g., phosphorylation of intracellular proteins, drug internalization, or protein trafficking) or late biological responses (e.g., cell proliferation, cell death or apoptosis, and induction of mRNA expression or cellular protein production). Although binding of drug to cell surface receptors can serve as an early assay endpoint for cell-based bioassays, it is more appropriate to categorize this assay format as a ligand-binding assay rather than a cell-based bioassay due to the lack of a cellular-response endpoint.

Cell-based NAb bioassays are most appropriate for certain biological drugs, particularly those with agonistic modes of action. However, this assay platform can be challenging due to its susceptibility to assay interference caused by subtle changes in cell culture conditions and sample matrix components. These causes of assay interference can substantially compromise assay precision, robustness, and sensitivity, ultimately hindering sensitive and precise measurement of NAbs in test samples. In contrast, CLBAs, especially immunoassay-based methods, are capable of overcoming these technical limitations inherent to cell-based bioassays, and they have, therefore, become another useful technology platform for NAb detection.

Neutralizing-antibody assays based on CLBAs are analytical procedures based on the competition between a labeled and an unlabeled ligand for a limited number of binding sites on a ligand-binding protein. As the concentration of unlabeled ligand increases, less labeled ligand binds to the ligand-binding protein and the measured response decreases. Thus the lower the assay signal, the more unlabeled ligand is detected in the sample. When NAbs bind a drug and inhibit its interaction with the drug target, this results in a change of assay response relative to a control sample that does not contain any neutralizing activity. Therefore, the NAb concentration is proportional to the percentage change of assay response. In theory, any ligand-binding assays based on immunochemical procedures, such as solid- or liquid-phase immunoassays, radioimmunoprecipitation assays (RIPA), and surface plasmon resonance (SPR), as well as cell-based binding assays, may be adapted to the CLBA format for detection of NAbs in test samples [10, 13–15].

10.3. COMPETITIVE LIGAND-BINDING ASSAYS BASED ON ELECTROCHEMILUMINESCENCE DETECTION

10.3.1. Electrochemiluminescence Detection System

Multiple detection systems, based on radioligand, enzyme-linked immunosorbent assays (ELISAs), chemiluminescence, and electrochemiluminescence assay platforms, are currently available for the development of immunoassays to analyze the specific interaction between antibody and antigen [10, 12–14]. Among these technology platforms, the electrochemiluminescence (ECL) detection system has become one of the most commonly used methodologies applied to immunogenicity testing.

Electrochemiluminescence methods utilize electrochemical reactions that produce luminescence in response to electric current stimulation [16, 17]. Ruthenium complexes are used to label the detection molecule in ECL immunoassays. The capture molecule, or the target, is immobilized on the electrode surface. When the ruthenium-conjugated detection molecule binds, directly or indirectly, to the capture molecule, the ruthenium label is immobilized near the electrode surface. Upon application of an electric potential across electrodes, ruthenium complexes near the working electrode and the co-reactant, tripropylamine (TPA), are oxidized, generating TPA radicals. A high-energy transfer then occurs from TPA radicals and excites the ruthenium complexes, which decay to the ground state following a normal fluorescence mechanism. The light is emitted at 620 nm and detected as the assay signal.

Recently, the ECL assay methodology has been used in conjunction with a flexible and high-throughput multiwell plate assay system incorporating carbon electrodes into assay plate well bottoms (Meso Scale Discovery, Gaithersburg, MD; see Fig. 10.1). This high absorbent carbon electrode surface facilitates the coating of capture molecules or even intact cells. The capture molecules, which are immobilized onto the plate surface, help bring the ruthenium-labeled detection molecules into close proximity with the carbon electrode surface. The system enables the ruthenium labels to be energized in the subsequent electrochemical reaction, generating an electrochemiluminescent assay signal.

10.3.2. Application of ECL Platform in CLBA-Based NAb Assays

Compared with traditional ELISA assays, the ECL assay method exhibits distinct advantages, including improved sensitivity, increased dynamic range, and better tolerance to sample matrix and excess drug in the circulation. Additionally, by requiring close proximity between the ruthenium labels and the electrode in order to generate the chemiluminescent signal, the ECL platform reduces the background derived from unbound detection molecules. This enables the assay to be conducted with limited wash steps, resulting in greater sensitivity to low-affinity ADAs, which are often not readily detectable in

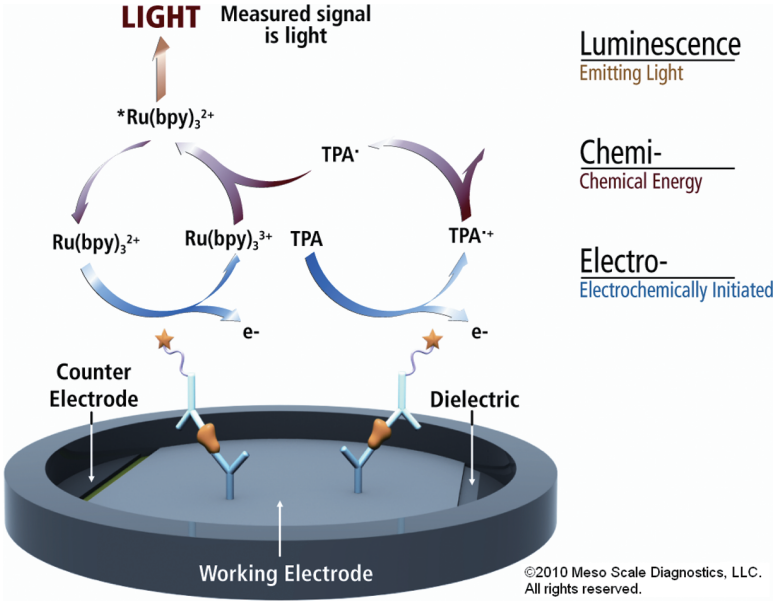

Fig. 10.1 Schematic illustrating the ECL detection methods developed by Meso Scale Discovery, LLC.

ELISA assays due to extensive washing [18]. These technical benefits provide the ECL assay platform enhanced value for immunogenicity testing.

Recently, the ECL technology platform has been applied in the development of CLBA-based NAb assays. The presence of NAb in test samples is detected through its competitive and measurable inhibition of the binding of drug target to the drug molecule. Based on this principle, different formats of ECL-based NAb assays have been developed.

10.3.2.1. Basic Principles for Assay Format Selection. While approaches based on risk level can guide general bioanalytical schemes for NAb assessment in clinical and nonclinical studies [2, 19], the selection of an assay platform for NAb detection relies more on a drug's mode of action.

Protein therapeutics exert their pharmacological actions by functioning as agonists or antagonists. **Agonists** directly bind to the receptors on the cell surface and trigger cellular response, whereas **antagonists** block cellular response either by inhibiting ligands from binding to their cell surface receptors or by binding directly to the receptors and blocking their biological functions. For therapeutic agonists or antagonists that directly bind and block the receptor function, the drug molecule exerts its functional activity directly on the cells. Therefore, the NAb activity can only be reliably assessed by a cell-based bioanalytical approach for this category of drugs. On the other hand, a range of antagonistic protein therapeutics, such as antagonistic monoclonal

antibody drugs, bind to soluble ligands, neutralizing their function without directly producing any functional activity on the cells. In this instance, a non-cell-based CLBA approach is appropriate for NAb assessment since these immunoassays reflect the drug's mode of action by measuring drug–ligand binding and the inhibition of such binding activity by NAbs.

10.3.2.2. Non-Cell-Based NAb CLBAs. Two assay formats are available for non-cell-based NAb CLBAs using the ECL technology platform. The **direct CLBA NAb assay** format is the simplest approach, based on measuring the binding of a drug to the ligand, while the **indirect CLBA NAb assay** format monitors drug-mediated interference of ligand–receptor binding. The two assay formats both demonstrate a drug's mode of action (antagonistic ligand binding) but measure the neutralizing activity at different assay endpoints.

10.3.2.2.1. Design of Direct CLBA NAb Assay Format. For the direct CLBA NAb assay format, the drug usually serves as the capture molecule and is immobilized on the carbon electrode surface, while ruthenium-labeled ligand (ruthenium–ligand) serves as the detection molecule, generating an electrochemiluminescent assay signal after it is anchored by the drug in close proximity to the carbon electrode surface. The neutralizing activity is assessed as the level of inhibition of drug–ligand binding by NAbs present in test samples (Fig. 10.2).

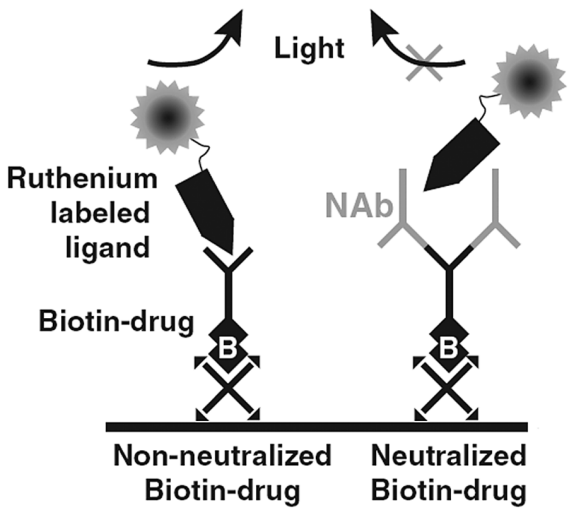

Fig. 10.2 Non-cell-based direct CLBA NAb assay format. The drug is conjugated to biotin, while the ligand is labeled by ruthenium. The biotin–drug is captured by streptavidin precoated on the carbon electrode surface and is detected by ruthenium–ligand, emitting an electrochemiluminescent assay signal. When NAbs are present, they competitively inhibit the drug from binding to the ligand, reducing the assay signal.

A reverse assay format appears to be possible using the ligand as the capture molecule and the drug as the detection molecule. However, this reverse format is much more prone to drug interference even if comparable assay sensitivity is achieved. Therefore, the direct CLBA NAb assay format using the drug as the capture molecule and ruthenium–ligand as the detection molecule should be the preferred approach for NAb assessment.

The direct CLBA NAb assay format requires that the drug be immobilized on the carbon electrode surface by direct coating or by capture via a linker (streptavidin or anti-species antibody). For direct coating, the unlabeled drug molecule is spotted directly on the assay plate. This approach precludes the need to conjugate the drug molecule to biotin and can be beneficial to proteins sensitive to chemical modification. However, due to the nonspecific manner of this coating approach, a portion of the directly immobilized drug molecules may adopt unfavorable conformations that mask critical epitopes required for ligand binding or recognition. In addition, this method could lead to higher variation when small volumes of the capture reagent are used for manual coating. Therefore, linker-mediated capture has become a more common approach for drug immobilization. In most cases, the carbon electrode surface can be precoated with streptavidin by the manufacturer, while the drug molecule is labeled by biotin (biotin–drug) and anchored to the plate surface through the specific interaction between streptavidin and biotin.

It is preferred that the direct CLBA NAb assays employ a fixed concentration of drug and ligand. The assay sensitivity of the direct CLBA NAb assay format is largely determined by the drug concentration selected for the NAb assay. Lower drug concentrations typically lead to more sensitive NAb assays. Therefore, it is important to optimize the drug concentration during assay development. Like cell-based NAb bioassays, the selected drug concentration ideally should yield 40–80% of the maximal assay signal generated through drug–ligand binding. This range should fall on the linear portion of the drug–ligand binding curve and provide a robust assay response. The low assay background typical of the ECL technology platform usually generates an adequate assay window with a high signal-to-noise ratio at the selected drug concentration. This allows a more flexible selection of drug concentration for NAb assays.

The following briefly describes a NAb assay procedure recommended for the direct CLBA NAb assay format using biotin–drug as the capture molecule and ruthenium–ligand as the detection molecule. The biotin–drug is first incubated with the properly diluted test sample to allow the binding of NAbs to the biotin–drug. The assay mixture is subsequently added to a streptavidin-coated assay plate, enabling the capture of the biotin–drug on the carbon electrode surface. After removal of unbound proteins by washing, the captured biotin–drug is detected by ruthenium–ligand, emitting an electrochemiluminescent assay signal after application of electric potential. When NAb binds to the biotin–drug, it competitively prevents the binding of ruthenium–ligand to the biotin–drug, reducing the assay signal. Hence, the concentration

of NAbs in the test sample is inversely proportional to the measured assay signal.

Based on the assay design, the essential components for this direct CLBA NAb assay format include biotin–drug, ruthenium–ligand, the test sample in the matrix of study species, and appropriate positive and negative controls. This simple assay format should allow rapid development and validation of robust and sensitive NAb assays across species for the immunogenicity assessment of antagonistic protein therapeutics to soluble drug targets.

10.3.2.2.2. *Design of Indirect CLBA NAb Assay Format.* An indirect CLBA NAb assay format can also be adapted for the detection of NAbs to antagonistic therapeutics neutralizing soluble ligands, based on drug-mediated interference of ligand–receptor binding. Compared with the direct CLBA NAb assay format, the indirect assay system introduces additional complexity to the biological interactions measured by the method. Specific consideration should be given to the challenges presented by the oligomeric receptors, which may be less likely to retain the original conformation necessary to bind to the ligand when not associated with the cellular membrane. In addition, cellular receptors typically have one or multiple transmembrane domains that need to be truncated to facilitate receptor purification. Therefore, concerns arise for these truncated ectodomains regarding proper protein folding and retention of structures necessary for ligand–receptor binding. The complexity of receptor purification further requires that lot-to-lot variation and stability of the protein products be effectively managed in order to maintain consistent assay performance.

One applicable format for an indirect CLBA NAb assay uses biotin-conjugated ligand (biotin–ligand) as the capture molecule and ruthenium-labeled receptor protein (ruthenium–receptor) as the detection molecule [20] (Fig. 10.3). Although the reverse format is also possible, it will not be discussed in detail in this chapter. The conjugation of biotin to ligand serves to facilitate a favorable orientation to bind to the receptor after the ligand is immobilized on the streptavidin-coated carbon electrode surface. The critical assay components include a fixed concentration of biotin–ligand, ruthenium–receptor, and the unlabeled drug, together with the test sample in the respective assay matrix and appropriate positive and negative controls. The assay is based on the inhibition of biotin–ligand binding to ruthenium–receptor by the drug and the restoration of ligand–receptor binding when the drug is neutralized by NAbs. The neutralizing activity in the test sample is thereby estimated by the level of restoration of ligand–receptor binding when the drug function is blocked by NAbs.

Based on the assay design, the indirect CLBA NAb assay format could be performed by first incubating the test sample with a fixed concentration of the unlabeled drug, followed by the addition of biotin–ligand to the assay mixture. The biotin–ligand is then captured by the streptavidin precoated on the carbon electrode surface and is detected by ruthenium–receptor, generating an elec-

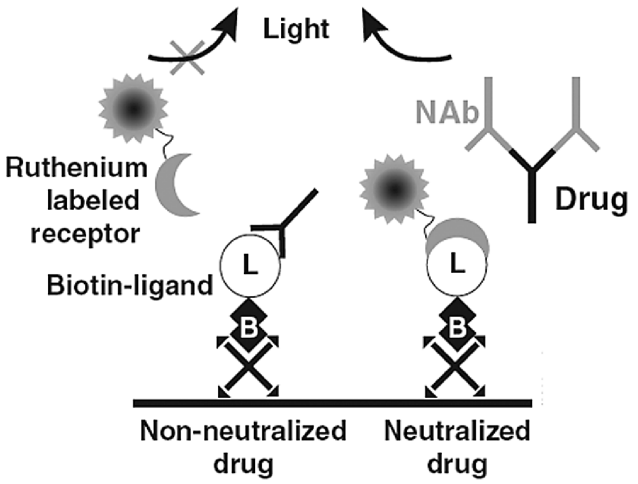

Fig. 10.3 Non-cell-based indirect CLBA NAb assay format. The ligand is conjugated to biotin, while the receptor is labeled by ruthenium. The biotin–ligand is captured by streptavidin precoated on the carbon electrode surface and is detected by ruthenium–receptor, emitting an electrochemiluminescent assay signal. Drug neutralizes the ligand, reducing the assay signal. When NAbs are present, they competitively inhibit the drug from binding to the ligand, restoring the assay signal.

trochemiluminescent signal. The drug binds biotin–ligand and prevents it from docking with the ruthenium–receptor, reducing the assay signal. When NAbs are present in the test sample, the neutralized drug is no longer able to bind to biotin–ligand and the assay signal is restored. The level of NAb activity is therefore proportional to the measured assay signal.

The assay sensitivity of the indirect CLBA NAb assay is dependent upon the ligand and drug concentrations. A lower ligand concentration will result in a lower drug concentration selected for the assay, which leads to a higher NAb assay sensitivity. The ligand concentration used for the NAb assay is ideally optimized to produce 40–80% of the maximal assay response generated through ligand–receptor binding. At fixed ligand concentration, the drug concentration should be selected to induce approximately 40–80% inhibition of the ligand–receptor binding and meanwhile maintain an appropriate assay window to support robust assay performance.

10.3.2.3. Cell-Based Binding Assays Using ECL Technology Platform. Cell-based bioassays are usually recommended for the detection of NAbs to therapeutic agonists or receptor-binding antagonists, utilizing cellular response as the assay endpoint. However, there are instances when the measurement of such an assay endpoint generates a limited assay window and/or imprecise assay results. Under these situations, cell-based binding assays using cells

expressing the receptor protein for ligand binding may be an alternative methodology for NAb assessment.

In 2006, Lu and colleagues reported the use of an ECL cell-based binding assay (Meso Scale Discovery, Gaithersburg, MD) as an alternative to a flow cytometry–based method and a cell-based ELISA [15, 21, 22]. One unique feature of this technology is the nonsmooth, high binding surface of the carbon electrode, which is able to bind not only adherent but also suspension cells without the use of any immobilizing agents. Compared with receptor proteins coated directly onto the electrode surface, the cell surface receptor protein is displayed in a more native and biologically relevant conformation for binding to its ligand. The ECL cell-based binding assay offers substantially higher throughput than flow cytometry. Compared with cell-based ELISAs, this ECL-based assay is easier to perform, offering better signal-to-noise ratio and a wider dynamic range [15]. Loyet et al. [23] used Jurkat cells transfected with a noncleavable tumor necrosis factor alpha (TNF-α) molecule in flow cytometry and ECL cell-based binding assays. The results demonstrated that the ECL cell-based binding assay appeared to be superior to flow cytometry at least with respect to assay throughput and the ability to better differentiate NAb responses between samples. These attributes demonstrate that the cell-binding ECL assay can be a useful technology platform for quantitative measurement of ligand binding to the cell surface receptor and therefore a potentially feasible assay methodology for NAb assessment.

10.3.2.3.1. *Design of Cell-Based CLBA NAb Assays.* The ECL cell-based binding assay measures the direct binding of biotin–drug to the receptor protein expressed on the cells immobilized on the carbon electrode surface. The biotin–drug is subsequently detected by ruthenium-labeled streptavidin (ruthenium–streptavidin). The presence of NAb activity is assessed through the inhibition or abolishment of the binding of biotin–drug to the target cells.

The following assay procedure is a summary of a representative approach used to develop a cell-based CLBA for a biological drug directly binding to a cell surface receptor [15,23]. An optimal number of cells expressing the target receptor are seeded in the assay plate, which features a carbon electrode built into the base of each assay well. The cells are incubated at room temperature to allow their steady attachment to the carbon electrode surface, followed by a blocking step. Meanwhile, the biotin–drug is incubated with the diluted test sample to enable the binding of NAbs to the biotin–drug. The neutralizing assay mixture is subsequently added to the assay plate for incubation with the cells. After removal of unbound molecules by washing, the biotin–drug captured on the cell surface is detected by ruthenium–streptavidin, producing an electrochemiluminescent assay signal. When NAb binds to the biotin–drug, it competitively prevents the binding of biotin–drug to the target receptor, reducing the assay signal. Hence, the concentration of NAbs in the test sample is inversely proportional to the measured assay signal.

Based on the assay design, the critical components of this method constitute a responsive cell line stably expressing a sufficient level of the target receptor, biotin–drug, ruthenium–streptavidin, the test sample in respective assay matrix, and appropriate positive and negative controls. Assay sensitivity is determined by the concentration of biotin–drug and the level of receptor protein expressed on the responsive cell line. As long as the assay window supports robust assay performance, a lower drug concentration and a cell line expressing higher levels of the target receptor should provide a more sensitive assay.

10.3.2.3.2. *Utility of Cell-Based Binding Assays versus Traditional Cell-Based Bioassays and Non-Cell-Based CLBAs for NAb Detection.* Although the ECL cell-based binding assays have been successfully applied for screening of ligands binding to cell surface receptors, this is still an emerging methodology for NAb detection. Compared with traditional cell-based bioassays intended to mimic the drug's physiological mechanism of action, cell-based binding assays tend to be less informative. In fact, this assay platform is, by nature, more similar to a ligand-binding assay rather than a cell-based bioassay measuring biological response to drug treatment. However, unlike a non-cell -based ligand-binding assay, the development of a cell-based binding assay can be more time consuming and the assay performance is highly dependent on the selected cell line. The washing necessary after every incubation step can lead to cell loss, limiting assay performance if the cells do not have sufficient affinity to the carbon electrode surface. Since the same technical limitations inherent to the traditional cell-based bioassays still apply, this often makes the cell-based binding assay format an inferior choice compared with a cell-based bioassay and the non-cell-based ligand-binding assays. Therefore, in most cases, the cell-based binding assay platform is an alternative format to consider only when the measurement of the performance characteristics of other assay formats generates suboptimal assay results. Comparison of cell-based binding assays to other NAb assay methodologies would further evaluate the pros and cons of this technology and its application in NAb assessment.

10.3.3. Positive and Negative Controls

Irrespective of the assay format used for the ECL-based CLBA NAb assays, positive and negative controls are critical for monitoring NAb assay performance characteristics, including assay sensitivity and the general operation of the assay system, during the entire term of a drug development program. Positive controls can be either monoclonal antibodies or purified polyclonal antibodies that neutralize the biological activity of the drug. If a polyclonal antibody is used for NAb assessment in clinical studies, it is preferable to obtain the antisera from nonhuman primates, which more closely mirror the human immune repertoire than other potential research species. Concentrations of the positive controls representing high and low assay response can be included in the assay. However, the "low" positive control is especially valuable, not only for monitoring assay performance, but also for ensuring that the

assay remains sensitive enough to detect any NAb response above the assay cut point. An appropriate concentration of low positive control should be chosen such that it is sufficiently close to the assay sensitivity, yet generates a low plate/run failure rate, such as 1% [24]. For the negative control, a drug-specific, non-neutralizing antibody is an ideal choice, although such negative controls are often not readily available. In these cases, pooled normal matrix derived from the study species is appropriate to serve as the negative control.

10.3.4. Interpretation of NAb Assay Results

Neutralizing-antibody assays can be designed as a yes-or-no type of qualitative screening assay or as a quasi-quantitative assay that measures the NAb activity. Detection of binding antibodies (NAb and non-NAb) with an ADA screening assay involves a conservative assay cut point to allow a 5% false positive rate [12, 25], and then a subsequent confirmatory assay to effectively eliminate the false positive samples and confirm true reactivity. Unlike such assays, NAb assays usually do not require additional confirmatory assays; the assay cut point can be computed to allow only a 1% false positive rate, which can help to minimize the reporting of false positives. This approach is justifiable for NAb assays because they are performed on samples previously screened and confirmed for the presence of binding ADAs.

For the non-cell-based direct CLBA (see Section 10.3.2.2.1) and the cell-based CLBA NAb assay formats (see Section 10.3.2.3.2), assay responses can be reported as a percentage inhibition of the maximal assay signal to reflect the level of NAb-mediated inhibition of drug–target binding. In these cases, the test samples lacking NAb activity produce near-maximal assay signal with corresponding percentage inhibition results below the assay cut point. The presence of NAb activity reduces the assay signal, generating percentage inhibition results above the assay cut point. For the indirect CLBA NAb assay format (see Section 10.3.2.2.2), the percentage restoration of the maximal assay signal can be used to express the NAb assay response. The restoration of assay signal results from NAbs competitively inhibiting drug–ligand complex formation and the subsequent restoration of ligand–receptor binding.

When interpreting the NAb assay results, it is important to take into consideration the potential interference from matrix components such as excess drug and soluble drug target in the circulation. The excess drug might obscure NAb detection by competing with the labeled drug to bind to NAbs, generating false negatives. Therefore, in accordance with the recommendations of Koren et al. [11], it is advisable that NAb-negative samples known to contain drug levels be reported as "negative" with an accompanying statement of possible drug interference. In addition, elevated levels of soluble drug target have been demonstrated to simulate NAb activity by binding to the drug, resulting in false positives. In these instances, strategies should be developed to identify these false positives and negatives, including assay procedures to remove the source(s) of assay interference.

10.4. COMPARATIVE ANALYSIS OF PERFORMANCE OF CELL-BASED BIOASSAYS AND NON-CELL-BASED COMPETITIVE LIGAND-BINDING ASSAYS

Immunoassays are considered to be less expensive than cell-based methods, to take less time to develop and validate, to offer higher throughput, and to be easier to transfer between laboratories. Hence, there has been a progressively greater focus on the use of immunoassays for NAb detection as an alternative to cell-based approaches. Two case studies are presented herein that directly compare cell-based bioassays to non-cell-based CLBA assays for detection of NAbs to therapeutic human monoclonal antibodies specific to chemokine (Case Study I) or cytokine (Case Study II). In both case studies, the non-cell-based CLBA NAb assays are immunoassays based on the ECL technology platform.

10.4.1. Case Study I—Direct Comparison of Performance Characteristics of the Two NAb Assay Formats

In this case study, the drug molecule is a human monoclonal antibody that serves as an antagonist blocking the biological function of a soluble chemokine molecule. The drug has been assessed with low risk of immunogenicity and minor anticipated clinical consequences. The cell-based bioassay utilizes DiscoveRx's PathHunter™ β-arrestin assay platform. The chemokine molecule stimulates its cognate receptor expressed on the surface of PathHunter™ HEK293 cells. The receptor is activated and followed by subsequent β-gal complementation, emitting a chemiluminescent assay signal. This assay signal is reduced when drug neutralizes the chemokine molecule. When NAbs are present in the test sample, they neutralize the drug, resulting in assay signal restoration. Therefore, the level of NAbs is directly proportional to the measured assay signal. The non-cell-based CLBA assay was based on Meso Scale Discovery's ECL technology platform. The biotin–drug is captured on streptavidin coated on the carbon electrode surface and detected by ruthenium-labeled chemokine (ruthenium-chemokine), emitting an ECL assay signal. NAbs competitively inhibit the binding of biotin–drug to ruthenium-chemokine, reducing the assay signal. As a result, NAb activity is inversely proportional to the measured assay signal.

A comparative analysis of the performance characteristics of the two assay platforms in Case Study I is briefly summarized in Table 10.1. A direct comparison of the relative sensitivities of the two assay formats was performed by evaluating a common panel of drug-specific monoclonal NAbs in both assay formats. The non-cell-based CLBA assay displayed greater sensitivity (<160 ng/mL) to all seven NAb controls evaluated in the assay, while two out of seven NAb controls were only detectable at concentrations greater than 500 ng/mL in the cell-based bioassay.

TABLE 10.1 Comparative Analysis of Cell-Based Bioassay and Non-Cell-Based CLBA in Case Study I

Assay Parameter	Cell-Based Biossay	Competitive Ligand Binding Assay
Minimal required dilution	1:10	1:10
Range of assay sensitivity	196–1248 ng/mL	102–160 ng/mL
Assay variability	Range of quantification 0.024–0.082 µg/mL	Range of quantification 0.008–0.178 µg/mL
Matrix interference	Lower assay signal recovery (18%–43%)	Higher assay signal recovery (94%–96%)
Drug tolerance	97.5 ng/mL	475.0 ng/mL
Assay specificity	Specific to NAb	Specific to NAb

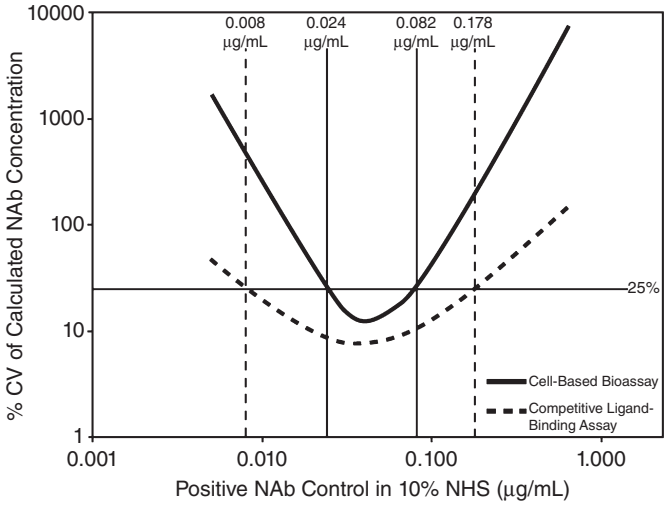

Fig. 10.4 Comparison of assay variability between the cell-based bioassay and non-cell-based CLBA. In Case Study I, assay variability was compared by statistical analysis of the data derived from NAb dose–response curves derived from cell-based bioassay and non-cell-based CLBA: (solid curve) data derived from the cell-based bioassay; (dotted curve) data derived from the non-cell-based NAb CLBA. For the cell-based bioassay, the range of quantification is 0.024–0.082 µg/mL in 10% pooled normal human serum (NHS). For the non-cell-based NAb CLBA, a wider range of quantification is observed, 0.008–0.178 µg/mL.

Assay variability was estimated for both assay platforms by statistical analysis of the data derived from multiple four-parameter logistic fitted NAb dose–response curves. The percentage coefficient of variation (%CV) of the calculated NAb concentration at a given assay response was computed and plotted against the corresponding nominal NAb concentration, generating nonlinear regression curves (Fig. 10.4). The range of quantification falls

TABLE 10.2 Evaluation of Matrix Interference of Cell-Based and Non-Cell-Based NAb CLBA in Case Study I

	Averaged Assay Signal Recovery (%)[a]		EC_{50} of NAb Dose Response Curve (ng/mL)[b]		Signal-to-Noise Ratio[c]	
Serum Concentration	Cell-Based Biossay	Competitive Ligand: Binding Assay	Cell-Based Biossay	Competitive Ligand-Binding Assay	Cell-Based Bioassay	Competitive Ligand-Binding Assay
0%	N/A	N/A	43.5	35.5	4.7	83.2
5%	43.1	95.6	39.0	38.5	5.4	52.9
10%	18.6	93.6	37.0	37.5	6.3	61.1
20%	18.3	93.8	52.0	42.5	6.9	172.2

[a]Averaged assay signal recovery for serially diluted NAb control in the presence of serum in the NAb assay.
[b]EC_{50} value indicates the NAb concentration in 10% normal human serum that reduces the assay response by 50%.
[c]The signal-to-noise ratio is represented by the ratio of maximal and minimal response derived from the NAb dose–response curve.

between the intersections of the nonlinear regression curves and 25% CV acceptance limit. Compared with the cell-based bioassay, the non-cell-based CLBA not only shows lower %CV at each data point but also demonstrates a wider range of quantification; a wider range of quantification generally correlates with lower assay variability.

In addition, for both assay formats, the NAb dose–response curves were not significantly impacted by the presence of serum, as indicated by the consistent EC_{50} values and signal-to-noise ratio (Table 10.2). However, the non-cell-based CLBA demonstrated better serum matrix tolerance with limited reduction of assay signal. In contrast, the cell-based bioassay was less tolerant to serum matrix. Significant reduction of assay signal was observed when increasing concentrations of serum were included in the assay. This discrepancy in serum tolerance is consistent with the observation that serum matrix can compromise the cellular response to drug or drug-inhibitable ligand treatment due to the presence of various interfering factors.

This study demonstrated that the non-cell-based CLBA is a superior choice compared with the cell-based bioassay because it exhibits higher assay precision, a greater dynamic range, and better serum matrix tolerance.

10.4.2. Case Study II—Direct Comparison of the Two NAb Assay Formats Applied in Clinical Sample Analysis

In this case study, the drug molecule is a human monoclonal antibody targeting a soluble human cytokine. Like Case Study I, the drug molecule is estimated

to be low risk in immunogenicity with minor anticipated clinical consequences. The cell-based bioassay uses WEHI cell line that responds to the cytokine and apoptoses in a dose-dependent manner. The assay endpoint is cell viability quantified by Cell Titer Glo™. The drug neutralizes the cytokine and increases cell viability, generating a chemiluminescent assay signal. When NAbs are present, they neutralize the drug and restore the killing effects of the cytokine on the WEHI cells, reducing the assay signal. The level of NAb is thus inversely proportional to the measured assay signal. The non-cell-based CLBA assay was based on Bioveris's ECL technology platform. Biotin–cytokine is coupled to streptavidin-coated magnetic beads and detected by ruthenium-labeled drug (ruthenium-drug), emitting an ECL assay signal. NAbs competitively inhibit the binding of ruthenium-drug to biotin–cytokine, reducing the assay signal. Therefore, the NAb activity is inversely proportional to the measured assay signal.

The two assay formats demonstrate comparable assay sensitivity and precision (data not shown) relative to the performance of control antibodies. Hence the two assay platforms were applied in clinical sample analysis to assess NAb activity in 24 binding ADA-positive samples. These samples were derived from a large clinical trial of human subjects treated with the monoclonal human antibody drug targeting the soluble inflammatory cytokine. Nine of the 24 samples were classified as NAb positive by the cell-based bioassay. In addition to those samples, however, the non-cell-based CLBA was able to detect NAbs in five more samples. Some of these five included samples classified as negative or inevaluable in the cell-based bioassay due to cell–serum incompatibility. These results indicate that the non-cell-based CLBA was more effective than the cell-based bioassay in the detection of NAb activity in clinical samples.

This study demonstrated that the non-cell-based CLBA was clinically more sensitive than the cell-based bioassay, and less affected by interference by matrix components.

10.5. SUMMARY

Although cell-based bioassays are the assay format recommended by regulatory agencies, non-cell-based CLBAs, such as ECL-based NAb assays, have become another viable option for NAb assessment. Comparative analyses have demonstrated that CLBAs show comparable or even superior assay performance than cell-based bioassays. Instead of using a blanket approach, this further facilitates a "fit-for-purpose" strategy for NAb assessment, allowing NAb assay formats to be determined based on drug's mechanism of action.

ACKNOWLEDGMENTS

We thank Meso Scale Discovery for sharing Figure 10.1, illustrating the electrochemiluminescence detection platform. We also thank Kenneth M. Goldberg

for statistical analysis support and Adrienne Clements-Egan for sharing some of the data.

REFERENCES

1. Schellekens, H. (2002). Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther*, *24*, 1720–1740.
2. Rosenberg, A.S., Worobec, A. (2004). A risk-based approach to immunogenicity concerns of therapeutic protein products. Part 1. Considering consequences of the immune response to a protein. *Biopharm Int*, *17*, 22–26.
3. Pendley, C., Schantz, A., Wagner, C. (2003). Immunogenicity of therapeutic monoclonal antibodies. *Curr Opin Mol Ther*, *5*, 172–179.
4. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelly, M., Anthony R., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
5. Benoit, P., Maguire, D., Plavec, I., Kocher, H., Tovey, M., Meyer, F. (1993). A monoclonal antibody to recombinant human IFN-cx receptor inhibits biologic activity of several species of human IFN-α, IFN-β, and IFN-ω. *J Immunol*, *150* (3), 707–716.
6. Schellekens, H., Casadevall, N. (2004). Immunogenicity of recombinant human proteins: causes and consequences. *J Neurol*, *251* (Suppl. 2), II/4–II/9.
7. Green, D. (2000). Spontaneous inhibitors to coagulation factors. *Clin Lab Haematol*, *22* (Suppl. 1), 21–25.
8. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.-J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.
9. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
10. Wadhwa, M., Bird, C., Dilger, P., Gaines-Das, R., Thorpe, R. (2003). Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. *J Immunol Methods*, *278*, 1–17.
11. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barret, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quanmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
12. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
13. Thorpe, R., Swanson, S.J. (2005). Current methods for detecting antibodies against erythropoietin and other recombinant proteins. *Clin Diagn Lab Immunol*, *12*, 28–39.

14. Wadhwa, M., Thorpe, R. (2008). Assessment of unwanted immunogenicity. In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Moller EH, eds. Springer, New York, pp. 57–73.
15. Lu, Y., Wong, W.L., Meng, Y.G. (2006). A high throughput electrochemiluminescent cell-binding assay for therapeutic anti-CD20 antibody selection. *J Immunol Methods*, *314*, 74–79.
16. Liang, P., Sanchez, R.I., Martin, M.T. (1996). Electrochemiluminescence-based detection of beta-lactam antibiotics and beta-lactamases. *Anal Chem*, *68*, 2426–2431.
17. Yang, H., Leland, J.K., Yost, D., Massey, R.J. (1994). Electrochemiluminescence: a new diagnostic and research tool. ECL detection technology promises scientists new "yardsticks" for quantification. *Biotechnology (NY)*, *12*, 193–194.
18. Liang, M., Klakamp, S.L., Funelas, C., Lu, H., Lam, B., Herl, C., Umble, A., Drake, A.W., Pak, M., Ageyeva, N., Pasumarthi, R., Roskos, L.K. (2007). Detection of high- and low-affinity antibodies against a human monoclonal antibody using various technology platforms. *Assay Drug Dev Technol*, *5*, 655–662.
19. Shankar, G., Pendley, C., Stein, K.E. (2007). A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. *Nat Biotechnol*, *25*, 555–561.
20. Gupta, S. (2005). Design and validation of a non-cell-based receptor binding assay for the detection of neutralizing antibodies to a biological therapeutic. IIR Immunogenicity Meeting.
21. Herzenberg, L.A., De Rosa, S.C. (2000). Monoclonal antibodies and the FACS: complementary tools for immunobiology and medicine. *Immunol Today*, *21*, 383–390.
22. Sedgwick, J.D., Czerkinsky, C. (1992). Detection of cell-surface molecules, secreted products of single cells and cellular proliferation by enzyme immunoassay. *J Immunol Methods 150*, 159–175.
23. Loyet, K.M., Deng, R., Laing, W.C., Wu, Y., Lowman, H.B., DeForge, L.E. (2009). Technology comparisons for anti-therapeutic antibody and neutralizing antibody assays in the context of an anti-TNF pharmacokinetic study. *J Immunol Methods*, *345*, 17–28.
24. Shankar, G., Devanaryan, V., Amaravadi, L., Barret, Y.C., Bowsher, R., Finco-Kent, D., Fischella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T., Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–1281.
25. Geng, D., Shankar, G., Schantz, A., Rajadhyaksha, M., Davis, H., Wagner, C. (2005). Validation of immunoassays used to assess immunogenicity to therapeutic monoclonal antibodies. *J Pharm Biomed Anal*, *39*, 364–375.

11

THE USE OF SURFACE PLASMON RESONANCE FOR THE DETECTION AND CHARACTERIZATION OF ANTIBODIES

Steven J. Swanson and Daniel Mytych

11.1. INTRODUCTION

The increase in the number of therapeutic proteins has resulted in attention focused on developing robust and reliable analytical methods to detect and characterize the presence of anti-drug antibodies (ADAs) that patients produce in response to treatment with these novel agents. Worldwide regulatory agencies are increasingly interested in the development and implementation of new analytical procedures to better detect and characterize antibodies to therapeutic proteins, as the presence of these antibodies can lead to safety and efficacy issues for patients.

There are several immunochemical methods available for the detection of binding antibodies to therapeutic proteins. The traditional enzyme-linked immunosorbent assay (ELISA), enzyme immunoassay (EIA), and radioimmunoprecipitation assay (RIP or RIPA) have all been well documented in the literature as proven methods for the detection of antibodies to protein drugs. Over the last few years, newer technologies have emerged. The electrochemiluminescence (ECL) technology has been introduced by Bioveris (previously IGEN) and, more recently, an ELISA-based format by Meso Scale Design (MSD; Gaithersburg, MD). Both the bead-based Bioveris [1] and the

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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ELISA-based MSD ECL testing platforms [2] have been successfully employed and accepted into the arsenal of immunochemical methodologies used by most in the biotechnology industry.

One of the available innovative technologies utilizes an instrument based on surface plasmon resonance (SPR) to detect and characterize antibodies directed against protein therapeutics. This immunoassay format has gained in popularity and has demonstrated its usefulness to detect antigen–antibody interactions [3–5]. A variety of commercial biosensors are available and have been reviewed [6], but the Biacore family of optical biosensor instruments is the most widely used in its class. Use of the Biacore biosensor for routine detection of ADAs was a logical extension of the detailed affinity and equilibrium constant determinations of antigen and antibody interactions. This immunoassay platform allows for direct measurement of binding activities as they occur. The therapeutic protein is immobilized and a serum sample suspected of containing antibodies against that therapeutic is allowed to interact with the immobilized therapeutic. If antibodies are present, they bind to the immobilized protein and that binding results in a signal that is proportional to the mass of the antibodies that have bound. This real-time detection allows the Biacore to detect low-affinity antibodies before they have the opportunity to dissociate. The SPR-based immunoassay has proven to be very reliable, specific, and sensitive for the detection of antibodies and is the preferred, currently available method for detecting low-affinity antibodies that are typical of an early antibody response. There are several publications that have demonstrated its usefulness for this purpose [7–11].

The purpose of this chapter is to provide guidance on how to implement the SPR-based systems, specifically the Biacore biosensor instrument, as a primary immunoassay platform or as a second-tier immunoassay to complement other common methods such as ELISA, RIP, and ECL for the characterization of anti-drug antibodies in the development of protein therapeutics. The general assay procedure using the SPR-based immunoassay involves the following steps:

1. Immobilize the therapeutic protein.
2. Regenerate the surface.
3. Inject the diluted serum sample (serum generally diluted to a final concentration of 5% through 50% into buffer containing soluble carboxymethyl dextran).
4. Monitor binding.
5. If binding is less then the threshold (or cut point), then the sample is negative for the presence of antibodies against the therapeutic protein.
6. If binding is greater than the assay threshold (or cut point), the sample may be positive; to confirm, add a confirmatory reagent that is an anti-human (or species-specific as appropriate) immunoglobulin reagent.

7. If binding of the confirmatory reagent is less than the confirmatory threshold (or cut point), the sample is negative.
8. If binding is greater than the confirmatory assay threshold (or cut point), the sample is positive.

The biotech pharmaceutical industry as well as the regulatory agencies that govern this industry have now accepted the use of SPR-based instruments such as the Biacore family of instruments. Therefore, it seems appropriate to describe the assay with a unique acronym. We propose the term "surface plasmon resonance immunoassay" (SPRIA) to describe immunological-type assays utilizing SPR-based systems for detection.

11.2. SUITABILITY OF SPRIA FOR DETECTION OF BINDING ANTIBODIES

There are several immunochemical methodologies available for the detection of all antibodies capable of binding the drug (binding antibodies). All methods and their corresponding detection instruments must meet three key criteria for selection. The method and its detection instrument must be validated, be compliant, and generate data that show reasonable correlation with other similar methods. The traditional ELISA, EIA, and RIP or RIPA have all been well documented in the literature as proven methods for the detection of antibodies to protein drugs. Over the last few years, newer technologies have emerged and have been successfully employed, such as the ECL technology, for example, MSD.

Before discussing how to implement the use of SPRIA to support immunogenicity testing during drug development, it must be made clear to the reader that this new technology platform has met the key requirements. The SPRIA for anti-drug antibody detection has been reviewed in numerous publications [1, 12–15]. The SPRIA meets many of the performance requirements for validated immunoassays, including limit of detection, accuracy, precision, specificity, and robustness. In addition, there are two validation parameters unique to this platform, immobilization range and ligand stability (see Fig. 11.1).

Because these immunochemical methods are used in support of regulated studies, that is, Good Laboratory Practices (GLP) and Good Clinical Practices (GCP), the instruments must meet minimum regulatory guidelines, including compliance with the (US) Code of Federal Regulations Part 11. The Biacore family of instruments has complied with these guidelines by providing users with a good practices GxP package encompassing installation and operational qualifications. In addition, a performance qualification kit can also be purchased to support long-range quality assurance. Most important, the use of the SPR-based instrument from Biacore for the detection of anti-drug antibodies is a proven and GxP-compliant analytical system. It is worth noting that we

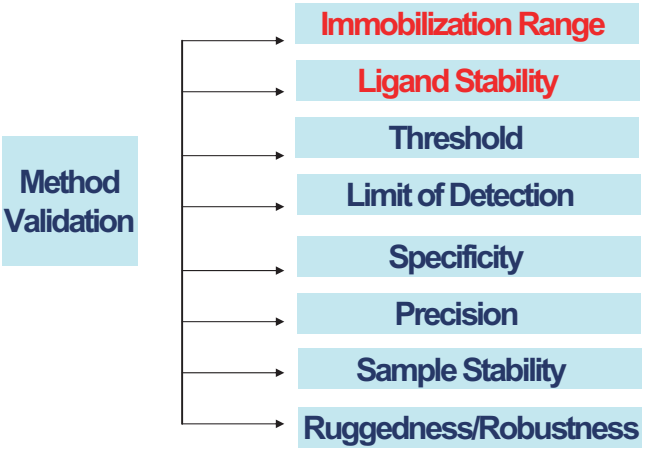

Fig. 11.1 The important parameters for an antibody assay validation are listed. Immobilization range and stability are unique to SPRIA.

support postmarketeing safety testing with a validated SPRIA that is an approved high-complexity assay governed by regulations under the Clinical Laboratory Improvement Amendments of 1988 (CLIA).

Finally, the correlation of clinical or preclinical data utilizing a validated SPRIA has also been extensively published [7, 15–17]. In fact, the SPR-based platform for the detection of ADAs against Vectibix™ detected a higher incidence of antibodies than the ELISA [18]. The real-time detection allows for the binding of the "low affinity" population of ADAs that would otherwise dissociate and not be detected when utilizing methods such as ELISA where multiple wash steps are required.

The selection of a biosensor immunoassay method to evaluate the immunogenicity of a human protein therapeutic is dependent upon many factors. In general, the SPRIA can be used for screening or to complement a screening ELISA or ECL-based immunoassay with a second-tier SPRIA to confirm and characterize an antibody response. In general, if the drug is not a fully human antibody (i.e., small protein less than 20,000 kDa, human Fc constructs or protein conjugates) we will initiate the development of a SPRIA as the screening assay. If the drug is a fully human antibody, we generally opt to develop a classical ELISA or use ECL technology as the screening assay. We have developed and validated SPRIA for the detection of anti-drug antibodies for all types of protein therapeutics at Amgen. The proteins varied from simple proteins and human Fc constructs to fully human antibodies. The success of the assay depends upon factors including the host, its inherent immunogenicity, and drug half-life to name a few. A common theme in immunogenicity testing is that the anti-drug antibody assay must use the "drug" to detect the serum antibody. We have found that when the protein drug is complex and if nonhuman sequences exist, it is extremely important to understand the specificity of

the antibody response, that is, what regions on the drug the antibodies are binding. The multi-flowcell cell capability of the Biacore instrument allows us to test a single diluted serum sample for binding to independently immobilized domains of the protein drug or to test for cross-reactivity between the drug and a homologous partner (endogenous protein) simultaneously, providing a wealth of information about the specificity of the antibody response.

In a preclinical setting, the IND-enabling toxicology studies required for human protein therapeutic drug development are performed in two species, typically using the rat, dog, mouse, or nonhuman primate. Based on guidance from a recently published paper on recommendations on immunoassays for antibody detection [19], the SPRIA meets all the described requirements. Within the preclinical setting, the administration of a human protein is expected to elicit an antibody response. In fact, the quality and quantity of ADA response is highly variable, producing both high- and low-affinity ADAs. The development and validation of a SPRIA has provided a platform that can readily detect the low-affinity ADA as well as the high-affinity ADA. These low-affinity antibodies can reach high concentrations in the circulation. They are generally not "neutralizing" but can act to sustain the drug in the circulation.

11.3. DEVELOPING THE ASSAY

Assay development using SPR can be accelerated compared with other immunoassay platforms due to the short assay times needed and the availability of real-time results. The first critical component of any SPR assay is determining a method to optimally immobilize the therapeutic protein drug onto the surface of the sensor. Covalent attachment of a protein to the sensor surface can utilize different coupling chemistries, the most common of which is amine chemistry. In general, the immobilization and the appropriate coupling chemistry used are dictated by the inherent properties of the protein, such as size, amino acid sequence, and the pI (pH at which it is charge neutral). Once the therapeutic has been immobilized, the availability of multiple epitopes to be bound by antibodies to the therapeutic protein, also described as the immunological reactivity, must be verified. The ideal immobilization is random such that the drug is immobilized in different orientations, with the end result being that all potential epitopes are available. If a therapeutic protein is immobilized in a site-specific way, that is, all the molecules are immobilized in the same orientation, it is likely that any antibodies that recognize an epitope masked by the immobilization would not be able to bind and would therefore not be detected.

Once appropriately immobilized, the next important component of the assay to consider is how to best regenerate the surface such that any component from the previous serum sample is removed, including any antitherapeutic antibodies. The reuse of a single drug surface is unique to the SPR-based

platform, also called the sample and regeneration cycle. The challenge inherent with this process is that different antibodies will have different affinity and avidity, some of which can be very difficult to remove. While a very strong regeneration solution, such as a high molar strength acid, will most certainly remove serum components such as ADAs from the sensor, they may also have an adverse effect on the immobilized therapeutic protein. The goal is to identify a regeneration solution that removes all bound serum components back to baseline while maintaining the immunological integrity of the immobilized therapeutic protein. There are a number of regenerations that can be utilized and the key to finding an appropriate solution lies in a full understanding of the stability of the therapeutic protein. Many regeneration solutions utilize 10 mM HCl. If this starting solution is not successful, the acid concentration can be increased, a salt solution such as NaCl can be added, and if necessary, a detergent solution can also be added. It is generally better to add short pulses of the regeneration solution rather than a single long exposure. With some proteins, it is better to use a basic regeneration rather than acidic. It is generally helpful to utilize a design of experiment matrix utilizing pH, ionic strength, and detergent choice as the parameters to evaluate. As described previously, the important parameters of binding capacity and baseline stability should be considered prior to finalizing a regeneration solution.

After multiple sample and regeneration cycles, it is expected that the binding capacity of the immobilized protein will diminish and the baseline will either accumulate mass on the immobilized drug or lose immobilized protein from the surface. Part of the process of identifying the optimal regeneration solution is to determine how many sample and regeneration cycles can be utilized before the performance of the immobilized protein drops below the acceptable level. As a reference, we have seen a range of supported sample and regeneration cycles from 30 to over 200.

Once immobilization and regeneration are worked out, attention is then focused on identifying the sensitivity of the assay. The process involves reducing background binding through appropriate buffer selection, sample dilution factor(s), and assay contact time. The goal is to minimize serum background binding while not inhibiting the binding of a specific antibody. One common approach to reduce background binding is to incorporate a soluble form of the carboxymethyl dextran from the sensor into the dilution buffer. This will promote nonspecific binding to the soluble form of the dextran in the buffer rather than to the dextran on the sensor. Increasing the dilution factor of the serum sample from 1:1 to 1:10 will reduce background binding, but of course it also reduces the specific binding as there are fewer ADAs available to bind. It may also be necessary to adjust the ionic strength or the pH of the buffer in order to minimize background binding. When evaluating the sensitivity of the assay, it is preferable to determine the sensitivity using both polyclonal and monoclonal antibodies where the affinity of the antibody is well established. This can help predict how successful the SPRIA will be at detecting antibodies with a range of affinities.

The final step in assay development is the final validation of the assay where all the parameters of the process can be determined. All of the traditional antibody detection assay parameters must be validated, including precision, accuracy (or sensitivity), linearity, effect of sample freeze–thaw, and specificity. It is generally recommended to follow the published guidelines on immunogenicity assay development and validation. In addition, the following unique SPR parameters must also be validated: baseline stability, immunological reactivity, and the number of sample and regeneration cycles allowed before a new sensor must be used. Validation allows an understanding of the performance of the SPR assay that is critical for the correct interpretation of results obtained from the assay.

11.4. VALIDATION OF THE ASSAY

11.4.1. Immobilization

Immobilizing the therapeutic protein is the first step in the development of an SPR assay. The goal of immobilization is to present the therapeutic protein in a random orientation such that the overall result is that all epitopes are available on the surface for binding by antibodies. The most common immobilization takes advantage of free amines by activating the surface with *N*-ethyl -*N*′-(3 dimethylaminopropyl) carbodiimide/*N*-hydroxy-succinimide (EDC/NHS) and most immobilizations take place at 25°C. In brief, the therapeutic protein is then added, followed by capping remaining reactive sites with ethanoalmine-hydrochloride. If this method proves unsuccessful, additional chemistries that can be used include sulfhydryl groups and carboxyl groups.

Once immobilized, it is important to verify that the positive control antibody is capable of binding to the newly immobilized protein prior to evaluating a regeneration solution. It can prove challenging to verify that multiple epitopes are available after immobilization, but it is an important parameter to determine. One way to test this is to use a series of monoclonal antibodies that bind to different regions of the therapeutic protein. If multiple epitopes are not available, it is possible that an important region of the therapeutic protein could be hidden or could have been structurally altered upon immobilization and is not available for binding. This would result in an entire population of antibodies not being recognized and the outcome would be false negative results. Most important, binding and neutralizing antibodies must be detected. An assessment of the control antibodies in a bioassay will ensure neutralizing antibodies are present.

The validation parameters around immobilization include the amount immobilized, the reproducibility of the immobilization, and the established range.

In general, a small protein (30 kDa) works well if immobilized within an immobilization range of 1500–3000 response units(RU). Larger proteins such

as immunoglobulins (Ig; 150 kDa) may require a 4000–6000 RU surface density to achieve adequate sensitivity. A range of ±1000 RU is a general guidance. One must remember to assess the nonspecific binding (NSB), sensitivity, and precision at the upper and lower end of the immobilization range. The extent of this validation parameter depends on the drug program. A protein drug in late-stage clinical development may require more robust assessment, incorporating immobilization over several days, on different instruments using different sensor chip lots.

11.4.2. Regeneration

During evaluation of regeneration solutions, the two most important parameters to consider are immunological reactivity of the surface and baseline stability. The immunological reactivity must be maintained so that any antibodies in the serum sample are still able to bind to the immobilized protein. Over time, the repeated use of regeneration solution will have the effect of breaking down or otherwise changing the inherent properties of the immobilized protein. After a certain number of sample and regeneration cycles, the surface will no longer be able to maintain binding by antibodies. The immunological binding can be determined by monitoring the binding obtained from a positive control antibody at two concentrations. The two concentrations would represent (1) a low antibody concentration that is slightly higher in concentration than the demonstrated sensitivity of the assay and (2) a high antibody concentration that is near the binding capacity of the surface to assess binding throughout the dynamic range of the assay. The low antibody concentration must demonstrate binding greater than the assay threshold (would be considered negative) to pass assay acceptance criteria. The high positive control must demonstrate consistent binding within ±10%. As a reference, the number of sample and regeneration cycles can vary between as few as 35 to greater than 200. The danger of not effectively monitoring the immunological binding and discontinuing analysis when the surface is exhausted is the high probability of falsely interpreting a low positive sample as negative.

The second important parameter is monitoring the baseline stability throughout sample and regeneration cycles. If the regeneration is incomplete, it is possible to experience a buildup of bound serum proteins, including antibodies against the therapeutic protein. If the baseline demonstrates gradual or rapid increases over time, which indicates the regeneration has not been complete, the surface may be compromised. The danger of continuing with additional sample analysis is the chance of falsely identifying a sample as negative. If there is a buildup of antibodies on the surface, all epitopes on the immobilized drug may be occupied due to the previous sample binding not being removed; therefore, the subsequent sample will not bind and be reported as negative. In contrast, if a secondary reagent is added to the bound sample to confirm its identity to be a human immunoglobulin, incomplete regeneration or accumulation will score the sample positive because the anti-human

immunoglobulin reagent is recognizing the built-up antibodies rather than antibodies from the sample just tested. This would result in a false positive sample result.

11.4.3. Assay Threshold

The assay sample threshold is determined once all the assay parameters have been established, including immobilization and regeneration. The goal is to determine the background binding of negative samples to the immobilized therapeutic protein. Once this range is established, it can be determined what level of binding represents a sample that contains antibodies against the therapeutic protein. A series of serum samples, ideally between 50 and 100, from the target population are diluted into sample analysis buffer and tested in the assay. It is important that only samples that are known to be negative be utilized in the determination of assay threshold. Once the samples are analyzed, a statistical evaluation should be made and any statistical outliers should be removed from consideration. If this important step is not included, the risk is that the threshold will be set artificially high so that some true positives may be falsely identified as negative. The remaining samples are used for the determination of the threshold. Basically, the 95% confidence interval is established and that value is the assay threshold. Any test sample that binds greater than the assay threshold is considered potentially positive. A key advantage of a SPRIA is the ability to confirm binding to be an antibody by the subsequent addition of an anti-species-specific antibody (see Fig. 11.2) prior to final assignment of positive status.

As an example, in human studies, the confirmatory reagent would be an anti-human immunoglobulin (Ig) reagent that is capable of binding to all classes and subclasses of human immunoglobulins. If addition of this reagent results in subsequent binding, the result of the assay is that the sample is positive for the presence of antibodies against the therapeutic protein. The level of subsequent binding necessary to trigger a positive result should be validated. A complexity with SPRIA is that, due to the real-time nature of the binding, the antibodies that were present in the sample and bound to the immobilized protein will begin to dissociate immediately after the binding reaction. This dissociation will produce a general downward trend in the baseline. This downward trend needs to be extrapolated and the subsequent binding of the confirmatory reagent evaluated against this extrapolated baseline.

Another aspect of the assay threshold that should be considered is that different populations may have different background binding. As an example, while healthy volunteers may demonstrate low levels of background binding, a population of patients that have an inflammatory condition may have much higher levels of background binding due to serum components including rheumatoid factor. When a population exhibits a different level of background binding, it can be discovered by comparing the binding of serum samples taken from patients prior to their receiving the first dose of the therapeutic protein

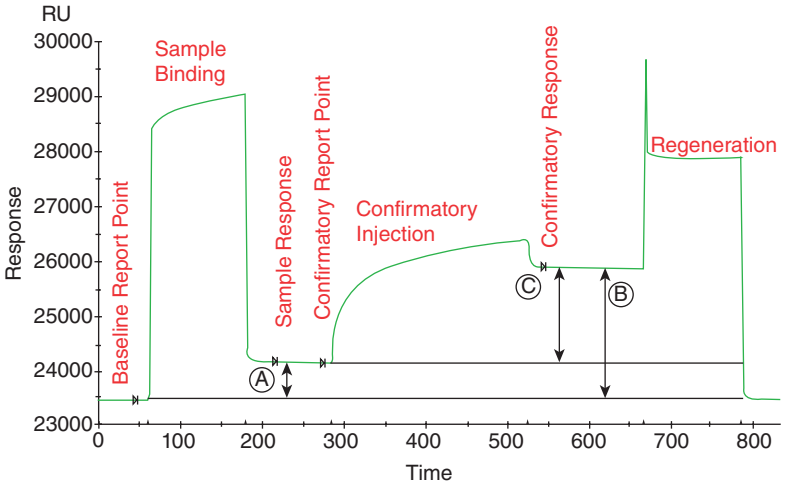

Fig. 11.2 A typical sensorgram. A refers to the difference between baseline and end of sample injection, B is the difference between confirmatory injection and baseline, and C is the difference between confirmatory injection and sample baseline established after sample injection. A is used to determine positive/negative; B is used for positive/negative when an assay relies on amplification for cut-point determination; and C is used to determine if sample binding is a result of an immunoglobulin.

(baseline samples). If the binding of baseline samples from a patient population is frequently above the assay threshold, the assay threshold may need to be adjusted. If the threshold is not adjusted, many samples will be falsely identified as positive.

To establish a study-specific threshold, the baseline samples from all the patients in the study are used to establish a new sample threshold. As with initial threshold determination, it is still important to remove statistical outliers prior to establishing the threshold. The successful determination of the assay threshold is critical to the overall success of any SPRIA.

11.5. CHARACTERIZATION OF POSITIVE ANTIBODIES

Once a sample is confirmed antibody positive against the therapeutic protein based on the initial SPRIA (see Fig. 11.3), there is an opportunity to characterize this antibody in a very simple and straightforward manner. There are many types of antibodies that can be generated in response to treatment with a therapeutic protein. Some of these antibodies have no significant clinical implications for the patient while others can be very problematic. The nature of an antibody response is that the immune response can mature over time. While early antibodies produced in response to a therapeutic protein tend to be of the IgM class, the immune response can mature into producing IgG antibodies.

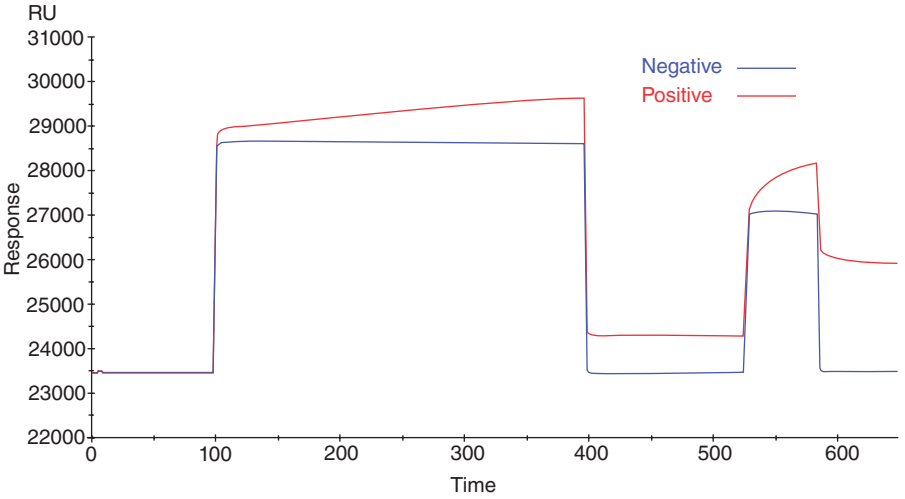

Fig. 11.3 A typical sensorgram from a positive and a negative sample. The positive sample demonstrates sample binding as well as binding of the confirmatory reagent. (See color insert.)

It is this immune response maturation that triggers a different type of antibody to be produced that is much more likely to have a meaningful clinical impact. IgM antibodies typically have a very low affinity and do not bind efficiently to the therapeutic protein. They also are not produced in a very high concentration. The low affinity coupled with the low concentration makes these antibodies less important clinically for the patient. These IgM antibodies are often not detected by antibody assays that do not utilize SPR. The reason is that low-affinity antibodies often dissociate from the immobilized protein prior to the final detection step. This dissociation is aided by multiple wash and incubation steps. While these early antibodies are not likely to induce a clinical effect, when trying to understand the immunogenicity of a therapeutic protein it is critical that all antibodies be identified so the immune response can be understood. Failure to detect these early antibodies results in an underestimate of the immunogenicity of the therapeutic protein.

The important characterization parameters to gain an understanding of the immune response include the proportion of bound antibodies that dissociate, the concentration of the antibody in the patient's serum, and the isotype of the antibodies present. Each of these can be readily determined using SPR. The final characterization parameter that is important to understand is whether the antibody neutralizes the biological effect of the drug. The best way to understand if the antibody is neutralizing is to perform a biological cell-based assay. This is not an evaluation that could routinely be performed using SPR. One exception could arguably be in the case of a monoclonal antibody that need only bind to its ligand to have a biological effect such as the case with a therapeutic antibody against a receptor. In this case, the mere act of blocking

binding of the therapeutic antibody to the receptor by an anti–therapeutic protein antibody would be sufficient to demonstrate biological activity.

11.5.1. Antibody Dissociation

The aggregate affinity with which ADAs bind to a therapeutic protein can help us understand the potential of the antibodies having a clinical effect. An antibody that binds very weakly and rapidly dissociates is less likely to remain bound to the therapeutic protein long enough to exert a lasting clinical effect. In patients that have a long-term immune response to a therapeutic protein, monitoring the aggregate affinity of polyclonal antibody response can provide insight into whether the immune response is maturing. Since the polyclonal response is a pool or aggregate of ADAs of mixed isotypes, affinity, concentration, and epitope specificity to the protein therapeutic, it is not possible to determine the aggregate affinity of the ADA. Since the ADA concentration(s) are variable, it is difficult to measure the aggregate association rate. Once the ADA is bound to the immobilized protein drug, the proportion of bound antibodies that dissociates can be determined by monitoring the response over a defined time. This is independent of the ADA concentration and allows for an assessment of the percentage of the polyclonal ADA response that contains high-affinity and low-affinity ADAs. An example of clinical data from subjects' antibody positive and demonstrating a slow, medium, and fast dissociation is shown in Figure 11.4. A rapidly dissociating antibody will yield a falling baseline. After a defined period of time, the percentage of bound sample that dissociated is then calculated. Positive control antibodies are traditionally

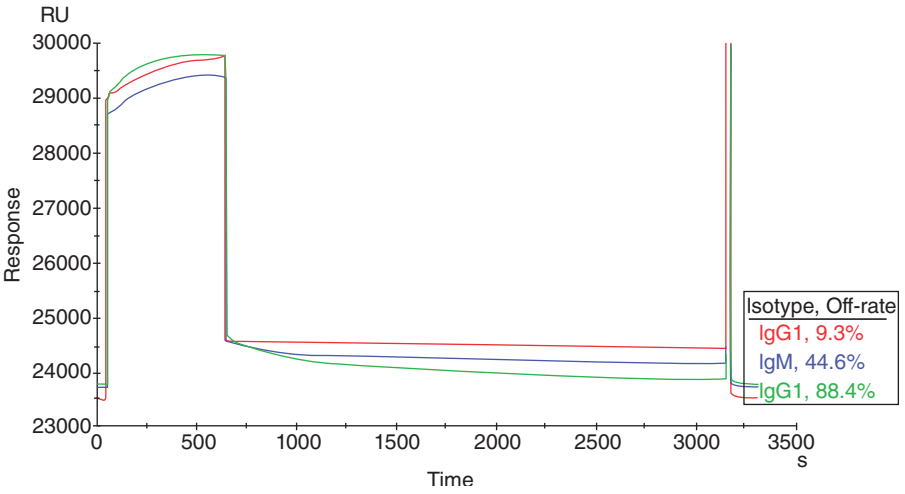

Fig. 11.4 This sensorgram illustrates differences in dissociation observed from antibodies with high, moderate, and low affinity. The low-affinity (rapidly dissociating) antibody is shown in green and the high affinity antibody is shown in red. (See color insert.)

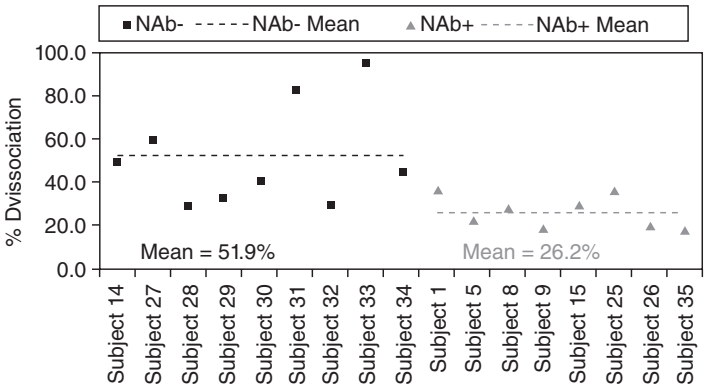

Fig. 11.5 Subjects with rapidly dissociating antibodies are in general less likely to have neutralizing antibodies compared with subjects with higher affinity antibody responses.

high-affinity, mature antibodies with very little dissociation. A rapid dissociation rate is indicative of a low-affinity antibody. It is important to note that when determining relative high-affinity versus low-affinity antibodies, SPR is only able to adequately capture the dissociation phase.

If the affinity of the antibody's binding is increasing, it suggests that maturation of the immune response is occurring. This could signal a shift from a nonclinically relevant antibody (large population of low-affinity ADAs) to one that has clinical relevance (high percentage of high-affinity ADAs). A case study demonstrating this is shown in Figure 11.5. It is of course important to recognize that the best indicator of a clinically relevant antibody is to monitor all aspects of how the therapeutic is working in a patient, including pharmacokinetics, pharmacodynamics, and clinical response.

While there is certainly great value in understanding the percentage of bound antibodies that dissociate, it is important to not over interpret these data. Because of the continuous monitoring of binding that is possible using SPR, these dissociation data can be collected. It is the lack of continuous monitoring that prevents platforms such as ELISA, radioimmune precipitation, and electrochemiluminescence to provide insight into the relative population of ADAs with a fast dissociation.

11.5.2. Antibody Concentration

The relative ADA concentration in a patient's serum sample can be readily determined using SPR. The amount of antibodies present in a serum sample can be determined by comparing the level of binding observed and comparing that with the level of binding obtained from a series of dilutions of the well characterized positive control antibody. It is important to note when using SPR to determine antibody concentration that the amount of antibody that is reported is always referenced or relative to the positive control antibody. The

positive control is acknowledged to be inherently different than the population of antibodies in the patient's serum sample. The positive control is a high-affinity, predominately IgG population of antibodies, while the patient likely has both IgG and IgM antibodies with different affinities. Because SPR assays base their binding on mass and the masses of IgG and IgM are quite different, it is acknowledged that comparing a patient sample with a positive control will not yield an absolute correct value for the amount of antibody present. Despite this concern, because SPR bases binding on mass and the same positive control can be used for an entire study, using the positive control as a reference does provide a solid anchor to allow comparison of binding of antibodies across a study. In addition, the use of the same reference also allows a clear idea of what is happening in any patient over time. If the SPR assay demonstrates increasing binding over time, it is highly suggestive of an increasing immune response that is likely to progress to having clinical relevance. In contrast, if the magnitude of the binding peaks and then decreases over time, it suggests a waning immune response that is less likely to have clinical relevance. Further, it is very likely that patients with a higher magnitude of binding antibodies are more likely to be patients where the immune response has clinical relevance and is associated with a neutralizing response (see Fig. 11.6). A case study shown in Figure 11.5 demonstrates a correlation of concentration with a clinical event. When determining the value of comparing the amount of binding from a sample with the binding of a positive control antibody, it is often useful to understand whether dilutions of the sample provide a parallel response when compared with the dilution of the positive control. The amount of antibodies produced in a patient is important to understand.

The other value of using SPR to determine the relative amount of antibody in a sample is to allow direct comparison with the sensitivity of the assay. An

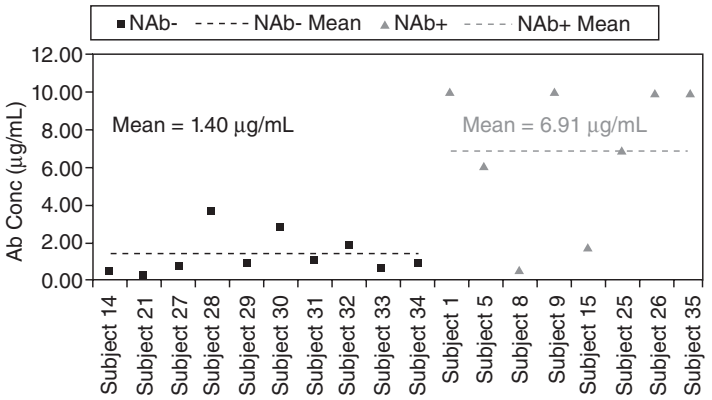

Fig. 11.6 Subjects with high concentrations of antibodies are more likely to have neutralizing and clinically relevant antibodies than subjects with low concentrations of antibodies.

antibody concentration value just slightly greater than the assay threshold is less likely to have clinical relevance than a value greatly above the assay threshold. Many investigators prefer to describe the magnitude of the immune response through the use of titer. A titer is the inverse of the dilution factor that still provides a positive result. The titer does not, however, allow direct comparison with the sensitivity of the assay.

11.5.3. Antibody Isotype

The value in understanding the composition of a patient's immune response is in knowing how the immune response is maturing. While early antibodies are typically IgM, further maturing of the immune response is accompanied by class switching that results in IgG antibodies. These antibodies can further mature from IgG1 through IgG4. Following the composition of the antibodies provides insight into the maturity of the immune response. These data can also help understand the likelihood of an immune response developing into one that has clinical relevance. For example, if a response never progresses from IgM to IgG, it is much less likely to have any clinical relevance. Most neutralizing antibodies are IgGs that have high affinity.

The identification of the ADA isotype(s) present in a patient's immune response to a therapeutic protein is also readily determined using SPR. Once the antibodies are captured on the immobilized surface, a series of isotyping reagents can be added in sequence to determine if that isotype is present. These isotyping reagents are based upon immunological differences in the Fc region of the antibodies. Since the Fc region is highly accessible when the antibody is bound to an immobilized therapeutic protein, isotyping is very effective. However, there are a couple of important considerations. First and foremost, the specificity of the isotyping reagents must be verified. It is insufficient to rely on manufacturers recommendations; it is critical to validate or qualify that these reagents do not cross-react with other antibody types and subtypes. One way to validate this using SPR is to immobilize purified IgG1 (for example) and then make certain that reagents that are reported to be specific for another subclass do not demonstrate binding to the IgG1. This should be repeated for each of the isotyping reagents that will be used, so that one would immobilize IgG2, IgG3, and IgG4 and confirm that only the specific isotyping reagents bind.

The second important consideration is to recognize that once an antibody class or subclass has been identified it may be necessary to regenerate, rebind the sample, and proceed with the other isotyping reagents. The presence of a positive isotyping response could cause a false result on a subsequent isotyping test. It is also important to consider that each isotyping reagent will have its own sensitivity. The sensitivity of the isotyping reagents is important when reporting results. For example, it would be preferable to report that the sample did not contain the minimum of X micrograms per milliliter of IgM rather than reporting the sample did not contain IgM.

11.6. CONCLUSION

This chapter has described some of the advantages as well as limitations of utilizing SPRIA to better understand the immunogenicity of therapeutic proteins. When deciding whether SPRIA is the best platform for detecting antibodies the important considerations include:

1. Can the therapeutic protein be successfully immobilized?
2. Does the immobilization allow presentation of multiple epitopes?
3. Can the surface be regenerated to prevent baseline drift and support a high level of binding capacity?
4. Does the assay have reasonable sensitivity such that meaningful antibodies are always detected?

If the previous questions can be answered satisfactorily, then the use of SPRIA is warranted. The advantages of this platform include superior detection of the early immune response and a superior platform for characterizing antibodies generated in patients against a therapeutic protein.

REFERENCES

1. Swanson, S.J., Jacobs, S.J., et al. (1999). Applications for the new electrochemiluminescent (ECL) and biosensor technologies. *Dev Biol Stand*, *97*, 135–147.
2. Moxness, M., Tatarewicz, S., Weeraratne, D., Murakami, N., Wullner, D., Mytych, D., Jawa, V., Koren, E., Swanson, S.J. (2005). Immunogenicity testing by electrochemiluminescent detection for antibodies directed against therapeutic human monoclonal antibodies. *Clin Chem*, *51* (10), 1983–1985.
3. Fagerstam, L.G., Frostell, A., et al. (1990). Detection of antigen–antibody interactions by surface plasmon resonance. Application to epitope mapping. *J Mol Recognit*, *3* (5–6), 208–214.
4. Malmborg, A.C., Michaelsson, A., et al. (1992). Real time analysis of antibody–antigen reaction kinetics. *Scand J Immunol*, *35* (6), 643–650.
5. Malmqvist, M. (1993). Biospecific interaction analysis using biosensor technology. *Nature*, *361* (6408), 186–187.
6. Baird, C.L., Myszka, D.G. (2001). Current and emerging commercial optical biosensors. *J Mol Recognit*, *14* (5), 261–268.
7. Mytych, D., La, S., Barger, T., Ferbas, J., Swanson, S. (2009). The development and validation of a sensitive, dual-flow cell, SPR-based biosensor immunoassay for the detection, semi-quantitation, and characterization of antibodies to darbepoetin alfa and epoetin alfa in human serum. *J Pharm Biomed Anal*, *49*, 415–426.
8. Wong, R.L., Mytych, D., et al. (1997). Validation parameters for a novel biosensor assay which simultaneously measures serum concentrations of a humanized monoclonal antibody and detects induced antibodies. *J Immunol Methods*, *209* (1), 1–15.

9. Takacs, M.A., Jacobs, S.J., et al. (1999). Detection and characterization of antibodies to PEG-IFN-alpha2b using surface plasmon resonance. *J Interferon Cytokine Res*, *19* (7), 781–789.
10. Alaedini, A., Latov, N. (2001). A surface plasmon resonance biosensor assay for measurement of anti-GM(1) antibodies in neuropathy. *Neurology*, *56* (7), 855–860.
11. Abad, L.W., Neumann, M., et al. (2002). Development of a biosensor-based method for detection and isotyping of antibody responses to adenoviral-based gene therapy vectors. *Anal Biochem*, *310* (1), 107–113.
12. Swanson, S.J., Mytych, D., et al. (2002). Use of biosensors to monitor the immune response. *Dev Biol (Basel)*, *109*, 71–78.
13. Wadhwa, M., Bird, C., et al. (2003). Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. *J Immunol Methods*, *278*, (1–2), 1–17.
14. Mire-Sluis, A.R., Barrett, Y.C., et al. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289* (1–2), 1–16.
15. Swanson, S.J., Ferbas, J., et al. (2004). Evaluation of methods to detect and characterize antibodies against recombinant human erythropoietin. *Nephron Clin Pract*, *96* (3), c88–c95.
16. Koren, E., Zuckerman, L., Mire-Sluis, A.R. (2002). Immune responses to therapeutic proteins in humans: clinical significance, assessment and prediction. *Current Pharmaceutical Biotechnol*, *3* (4), 349–360.
17. Tatarewicz, S., Wei, X., Gupta, S., Masterman, D., Swanson. S.J., Moxness, M. (2007). Development of a maturing T-cell-mediated immune response in patients with idiopathic Parkinson's disease receiving r-metHuGDNF via continuous intraputaminal infusion. *J Clin Immunology*, *27*, 620–627.
18. Lofgren, J., Dhandapani, S., Pennucci, J., Abbott, C., Mytych, D., Kaliyaperumal, A., Swanson, S.J., Mullenix, M.C. (2007). Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *J Immunol*, *178* (11), 7467–7472.
19. Ponce, R., Abad, L., Amaravadi, L., Gelzleichter, T., Gore, E., Green, J., Gupta, S., Herzyk, D., Hurst, C., Ivens, I., Kawabata, T., Maier, J., Mounho, B., Rup, B., Shankar, G., Smith, H., Thomas, P., Wierda, D. (2009). Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies. *Regul Toxicol Pharmacol*, *54*, 164–182.

12

HYPERSENSITIVITY REACTIONS TO BIOPHARMACEUTICALS: DETECTION AND QUANTIFICATION OF DRUG-SPECIFIC IGE ANTIBODIES

Jörgen Dahlström and Lennart Venemalm

12.1. INTRODUCTION

Hypersensitivity reactions to biopharmaceutical drugs can be conveniently subdivided into immediate reactions occurring rapidly within a couple of hours and delayed reactions appearing from 6 hours to several days after infusion [1]. Immediate reactions may have different pathomechanisms, including allergic immunoglobulin E (IgE)-mediated reactions that involve cross-linking of IgE on mast cells and basophils. Other mechanisms include the formation of IgG–antigen complexes and activation of the complement system.

Delayed reactions can be subdivided into IgG-mediated and T-cell-mediated reactions. The formation of IgG antibodies against biologicals occurs frequently and does not necessarily cause hypersensitivity reactions. The main effect of IgG antibodies against biologicals is neutralization of the biological activity, which may result in treatment failure.

Immunoglobulin E antibodies mediate allergic hypersensitivity reactions and are present in serum at the lowest concentration of the five immunoglobulin subtypes (approximately 0.0005% of total serum immunoglobulins in

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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adults) [2]. IgE binds to the surface of mast cells and basophils with high affinity and initiates immediate hypersensitivity reactions by binding to polyvalent antigens, thereby releasing various mediators (histamine, tryptase, leukotrienes, prostaglandins, etc.). Importantly, the IgE system is very sensitive and is able to react to very small amounts of antigen.

Although many acute reactions to biologicals, for example, infusion reactions, do not usually seem to be IgE-mediated, a number of anaphylactic reactions involving IgE have been reported. It is important to distinguish between infusion reactions that are IgE-mediated and those that are not, since IgE-mediated events seem to have a rapid and more severe onset [3]. It may not be possible to distinguish clinically between IgE-mediated and non-IgE-mediated reactions, and laboratory tests for measuring IgE antibodies against biologicals are therefore of diagnostic value.

Anaphylactic reactions against the chimeric monoclonal antibody (mAb) cetuximab have been proven to be IgE-mediated [4] and are to date among the most prominent examples of IgE-mediated hypersensitivity reactions to a biopharmaceutical drug. In most subjects with hypersensitivity reactions to cetuximab, anti-cetuximab IgE antibodies were present in serum before therapy. In other cases, IgE antibodies against the allergen are not preformed and develop during the course of therapy.

12.2. IMMUNOGLOBULIN E–MEDIATED REACTIONS TO BIOPHARMACEUTICALS

The dramatic increase in the use of recombinant monoclonal antibodies and other biopharmaceuticals in the treatment of cancers and autoimmune diseases, such as inflammatory bowel disease and rheumatoid arthritis, has resulted in increased reporting of hypersensitivity reactions, including anaphylaxis. This trend is expected to continue as new agents enter the market.

Immunoglobulin E–mediated reactions can cause urticaria, angioedema, bronchospasm, and anaphylaxis. Anaphylaxis is a severe, life-threatening, generalized or systemic hypersensitivity reaction [5]. The potentially life-threatening nature of anaphylactic reactions makes it necessary to confirm the clinical diagnosis and identify the triggering agent in order to avoid reactions in the future.

Anaphylaxis is characterized by an acute illness (onset occurs in minutes to several hours) that follows after exposure to an allergen. Target organs include skin and mucosal tissue (e.g., urticaria, pruritus, flushing, angioedema), respiratory tract (e.g., dyspnea, wheeze–bronchospasm), heart and vasculature (e.g., hypotension, myocardial infarction, arrhythmias), and gastrointestinal system (e.g, cramps, abdominal pain, vomiting). Anaphylaxis can be classified as allergic or nonallergic depending on whether the reaction is caused by an immunologic mechanism or not. Allergic anaphylaxis may be IgE-mediated or non-IgE-mediated, the latter being caused by IgG immunocomplex,

complement-related, or immune cell-mediated mechanisms [5]. Mechanisms that do not involve IgE are both less common and less well defined [6].

Infusion reactions are typically mild to moderate in intensity but may also present with symptoms of severe hypersensitivity reactions such as bronchospasm, hypotension, urticaria, and cardiac arrest. These events have resulted in warnings in the prescribing information for biopharmaceutical products. The exact mechanism for non-IgE-mediated infusion reactions to biologicals is not known, but they may in part be induced by cytokine release [4]. The overlapping symptoms for classical infusion reactions and IgE-mediated anaphylaxis make it important to use laboratory tests in order to distinguish between the IgE- and non-IgE-mediated events. This is especially true since approaches for avoiding future reactions differ depending on the mechanism of reaction. Thus, some infusion reactions may be avoided by a slower rate of infusion, which is not a possible strategy for IgE-mediated reactions. Premedication with corticosteroids and antihistamines may also be used to prevent or dampen non-IgE-mediated reactions [4]. Furthermore, predictive tests may also be used to determine which patients with severe infusion reactions can be safely managed with rechallenge. Finally, IgE testing may also be used as a predictive indicator to determine which patients are at the highest risk of severe IgE-mediated hypersensitivity reactions, such as the detection of IgE antibodies against cetuximab. Some examples of IgE-mediated or possibly IgE-mediated immediate reactions to biopharmaceutical drugs are described in the following subsections. This survey is not intended to be comprehensive and a number of biologicals with well-known risks of IgE-mediated reactions such as, for example,. insulin, enzymes, and tetanus toxoid have not been described here.

12.2.1. Cetuximab

Cetuximab is a chimeric mAb directed against the epidermal growth factor receptor and is used to treat colorectal cancer and squamous cell cancer of the head and neck. It is well known for causing anaphylactic reactions, especially in some southern regions of the United States [4]. Severe hypersensitivity reactions to cetuximab occur in 3% of patients, while 22% of patients who were treated with cetuximab in Tennessee and North Carolina had severe hypersensitivity reactions. Most of these reactions occur within minutes upon first treatment exposure and have been shown to be IgE-mediated. Of 25 patients with a hypersensitivity reaction, 17 had a positive test for IgE antibodies against cetuximab in pretreatment serum samples. In contrast, only 1 of 51 subjects who did not experience a hypersensitivity reaction had IgE against cetuximab. IgE antibodies were found in 21% of control subjects in Tennessee and in only 0.6% of subjects from Boston, which correlates with the prevalence of hypersensitivity reactions to cetuximab. IgE antibodies were measured with the use of ImmunoCAP® (Phadia U.S.). ImmunoCAP is an assay based on antigen solid-phase binding of specific IgE antibodies, which are detected with

a secondary enzyme-labeled anti-IgE antibody [7]. The IgE assay showed a sensitivity of 92% for patients having experienced a severe hypersensitivity reaction, compared with 68% for patients with any hypersensitivity reaction [4]. Assay specificities were 90% and 92%, respectively.

It has been shown that anti-cetuximab IgE antibodies are specific for the carbohydrate galactose-α-1,3-galactose (α-gal), which is present on the Fab portion of the murine heavy chain at position 88. Galactose-α-1,3-galactose is present on tissues from nonprimate animals and it has been shown that a novel and severe food allergy from eating beef, pork, or lamb has been associated with IgE antibodies against α-gal [8]. Researchers are currently investigating the possibility that bites from ticks or tick larvae are responsible for triggering the production of IgE antibodies against α-gal.

Cetuximab is a prime example of the unpredictable nature of hypersensitivity reactions to biopharmaceutical drugs. Naturally, we expect antibodies to be directed against a mouse amino acid sequence of therapeutic mAbs. In contrast, IgE antibodies against cetuximab were instead directed against an oligosaccharide present on nonprimate mammalian proteins.

12.2.2. Infliximab

Infliximab is a chimeric mAb directed against tumor necrosis factor alpha (TNF-α), which plays a central role in inflammatory regulation. Infliximab is used in the treatment of autoimmune diseases such as fistulizing Crohn's disease, ulcerative colitis, psoriasis, and rheumatoid arthritis.

Acute infusion reactions occur at a rate of 3–5% of treated patients and can usually be reduced by slowing the infusion rate [9]. Anaphylaxis has been reported to occur in some patients, although the exact mechanism has not been fully defined. It has been shown that antibodies to infliximab are mainly of the IgG type. However, an Italian research group has detected anti-infliximab IgE antibodies in 3 out of 11 patients with hypersensitivity reactions [10]. These patients were also skin test positive to infliximab. Antibodies were detected in sera collected during the second course of treatment after the first infusion. In contrast to cetuximab, IgE antibodies were not detected in sera before treatment, ruling out the possibility of pretreatment sensitization. Interestingly, a number of patients with severe reactions were also shown to have anti-infliximab IgM antibodies.

12.2.3. Adalimumab

Adalimumab, a fully humanized mAb, is a TNF-α blocking agent that is used against a number of autoimmune diseases. In clinical trials, allergic reactions have been observed in approximately 1% of patients [11]. Cutaneous injection site reactions are reported to occur in 15% of patients and are often cell mediated. However, immediate IgE-mediated reactions to adalimumab have recently been reported in two patients, demonstrating the role of IgE in some

patients receiving adalimumab therapy [12]. The IgE-mediated mechanism of these reactions was demonstrated by positive skin testing and histamine release assay performed on peripheral blood leukocytes from the patients. Furthermore, a passive transfer experiment with serum from one of the patients to basophils from a healthy nonatopic control sensitized those cells to release histamine on exposure to adalimumab.

12.2.4. Etanercept

Etanercept is a fusion protein composed of two binding regions of the human TNF-α receptor p75 that is used for the treatment of rheumatoid arthritis, psoriasis, and ankylosing spondylitis. Injection-site reactions are frequent [13] and urticaria and angioedema are occasionally reported. Recently, two cases with injection site reactions were reported as being immediate [14]. Intradermal tests in these patients were positive at immediate reading and negative at late reading, suggesting an immediate, possibly IgE-mediated, reaction.

12.2.5. Omalizumab

Omalizumab is a humanized mAb against the Fc-region of IgE for use in allergic asthma. Omalizumab-induced anaphylaxis has been reported to occur in approximately 0.1% of patients [15]. Most of the reactions occurred in the first two hours after one of the first three doses, including the first dose. In another report, 124 cases of anaphylaxis to omalizumab were reviewed and categorized by time-to-onset and other clinical features [16]. Thirty-nine percent of the cases were first-dose reactions and time-to-onset of anaphylaxis varied from minutes to days. Some patients also experienced a protracted progression of symptoms. Based on these cases, in 2007 the US Food and Drug Administration (FDA) ordered a black box warning for omalizumab [17]. In two cases, the excipient polysorbate has been suspected to be the causative agent for the hypersensitivity reactions observed following omalizumab therapy [18].

12.2.6. Daclizumab

Daclizumab is a humanized monoclonal anti-Tac antibody that functions as an antagonist against the interleukin-2 receptor of T-cells. It is used to prevent rejection in organ transplantation, especially kidney transplantations. Severe, acute (onset within 24 hours) hypersensitivity reactions, including anaphylaxis, have been observed both on initial exposure to daclizumab and following re-exposure [19].

12.2.7. Basiliximab

Basiliximab is a chimeric mAb that targets the α-chain of the high-affinity interleukin-2 receptor complex. Severe acute hypersensitivity reactions have

been reported, and the presence of anti-basiliximab IgE has been demonstrated by enzyme-linked immunosorbent assay (ELISA) [20].

12.2.8. Muromonab

Muromonab CD3 is a monoclonal murine antibody that binds and blocks the CD3 complex of the T-cell receptor and is used to reverse renal graft rejection. Anaphylactic hypersensitivity reactions are known to occur, and IgE-antibodies have been detected in sera from a number of patients [21, 22].

12.2.9. Natalizumab

Natalizumab is a recombinant humanized IgG4κ mAb anti-α_4-integrin used in multiple sclerosis treatment. In 2010, an IgE-mediated mechanism was demonstrated in a case report [23]. The patient reacted immediately after the second administration of the drug, and immunoblotting, positive intradermal skin test, and a fairly high concentration of anti-natalizumab IgE antibodies were evidence of an IgE-mediated reaction.

12.3. PREREQUISITES AND DEMANDS ON A CLINICAL IGE TEST

12.3.1. Assay Strategies to Determine Immunogenicity

A sound strategy for assessing the immunogenicity of biological products is essential. A screening assay for identification of antibody-positive samples or patients should be the first step in this strategy. As a second step, an analytical immunochemical assay should be developed to confirm and, if possible, quantify the findings in the screening assay and determine antibody specificity. Finally, functional assays for assessing the neutralizing capacity of antibodies could be developed.

12.3.2. Allergic IgE-Mediated Responses Must Be Addressed in Clinical Development of Biopharmaceuticals

Since biopharmaceuticals are often administered repeatedly over an extended time, secondary humoral responses of the IgE isotype could be facilitated. This makes it important to consider IgE responses to the biological as a possible risk for causing anaphylactic reactions and thus highlights the importance of IgE assay development in clinical drug characterization. The value of developing assays that discriminate between antibodies of specific isotypes is also stressed by regulatory authorities. Allergic immune responses to therapeutic biopharmaceuticals span from clinically unimportant as seen in growth hormone (GH)-lacking children, where antibodies to recombinant human GH (anti-rhGH) were formed without any effect on the growth rate of the children [24], to reactions that pose a serious threat to patient health. Such varied

responses necessitate a thorough documentation of immunogenicity during the drug development process, to demonstrate the safety of the new drug. Thus, it is critical to develop robust, sensitive, and validated assays to determine immunogenicity.

12.3.3. Challenges in Setting Up IgE-Detecting Assays

The design of a human IgE detection assay can often be based on a pre-existing IgG assay. Since IgG immunogenicity assays are usually developed early, during the preclinical phase, drug development guidelines exist for the design, validation, and performance specifications of assays for detecting and quantifying human IgG antibodies specific for new biotherapeutics [25–28]. Developing an assay to detect human IgE antibodies against a biopharmaceutical is, however, more complex than developing the corresponding human IgG antibody assay. There is rarely any human IgE available to be used as a control during early preclinical or clinical phases, and animal antisera are not useful in developing human antibody assays. Other challenges in IgE assay development are the low concentration of IgE compared with IgG (nanograms per milliliter compared with microgram-per-milliliter levels) present in human serum, necessitating exceptional assay sensitivity. Furthermore, the presence of high concentrations of IgG antibodies of the same specificity can interfere with the IgE anti-drug assay. However, the latter point may be addressed by having an excess amount of allergen present in the test, in a dedicated IgE assay such as the noncompetitive heterogeneous immunometric assay discussed later in this chapter, or possibly by IgG depletion as described in the protocol by Fu et al. [29].

12.3.4. Steps in Assay Development

Several issues must be addressed when setting up an *in vitro* assay to be used in clinical trials of a new biopharmaceutical. These include deciding on the type of specimen and assay format, and formulating a strategy for developing the assay.

12.3.5. Choice of Test Specimens

Serum and plasma are considered the normal test specimens for the drug-specific IgE antibody assays discussed in this chapter. Alternatives such as tear fluid, sputum, and cerebrospinal fluid are primarily used in research.

12.3.6. Possible Assay Formats for *In Vitro* Determination of IgE Specific for a Biological Drug

Several possible assay formats exist and are described in more detail in other chapters; a brief comparison of these will be discussed here.

12.3.6.1. Competitive ELISA to Detect All Drug-Specific Antibodies. In this assay format, the drug is first coated on a solid phase, usually the wells of a microtiter plate, and then a conjugated drug-specific reference antibody is added at a specific concentration that results in a positive signal. The reference antibody can be labeled with, for example, biotin, fluorescence, or a radioactive isotope. Thereafter, increased concentrations of unconjugated antibody or the same reference control antibody are added to the test serum. This results in dose-dependent inhibition and the detected response decreases. Importantly, this assay does not discriminate between IgE and IgG or other isotypes of human antibody and it is therefore not useful for detecting drug-specific IgE antibodies. In this assay, the reference antibody needs to be carefully characterized to ensure that it is relevant to the samples of anti-drug antibody. Further, the conjugated antibody must be characterized; first, to determine the ratio of conjugated antibodies, and second, to confirm that the immunoreactivity (binding affinity, specificity) is not altered by the conjugation.

12.3.6.2. Surface Plasmon Resonance Assay. Surface plasmon resonance (SPR) is used in biosensor systems to enable direct real-time measurement of molecular interactions. Usually SPR biosensors have one of the binding molecules bound to the surface of the sensor chip; then the other potentially binding molecule, in solution, is injected over the chip. Binding causes changes in mass on the sensor chip surface, which are detected by changes in refractive index close to the sensor chip surface. The response is proportional to the number of molecules bound to the chip surface. If used for the detection of drug-specific IgE, the drug is bound to the chip; if antibody is present in added samples, the resonance values will change. To ensure that the bound antibody is of the IgE isotype, anti-IgE is added, which should result in a response. In addition to the detection of drug-specific IgE antibodies, the SPR assay can give information on kinetic rate constants, antibody concentration in samples, and affinity constants that can be used for qualitative or semiquantitative determinations.

12.3.6.3. Noncompetitive Heterogeneous Immunometric Assay Format with the Drug on Solid Phase. The radioallergosorbent test (RAST) was introduced so that IgE antibodies directed against a specific allergen could be detected [30]. This assay had the allergen insolubilized on a paper disc (allergosorbent) to which antibodies of all isotypes from the sample would bind. The next step in the assay procedure was to separate free from bound antibody; finally, radiolabeled anti-human IgE was used to detect IgE antibodies that had bound to the antigen. The RAST format has been modified with the use of other solid phases that have a higher capacity to bind allergen, such as ImmunoCAP. Recently, monoclonal anti-human IgE antibodies labeled with nonisotopic labels have been used instead of polyclonal antibodies coupled with a radiolabel. This modified RAST is considered the design of choice when developing a drug-specific IgE antibody assay (Fig. 12.1).

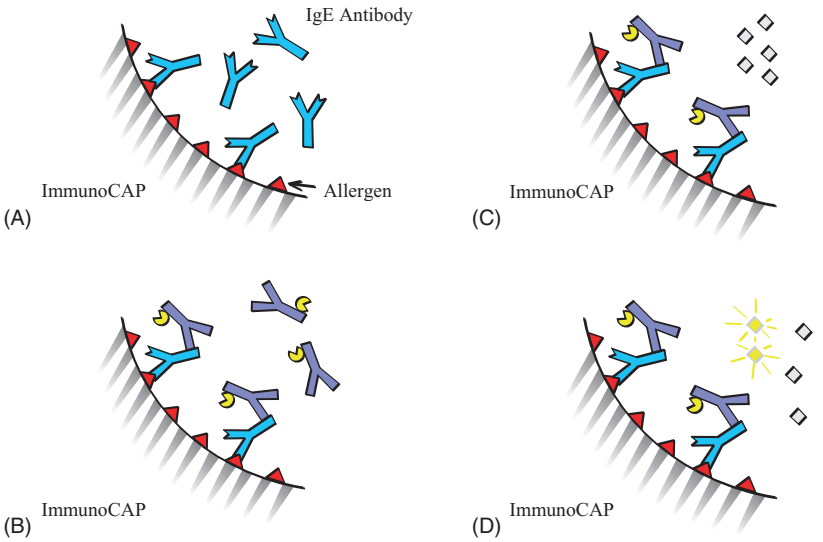


Fig. 12.1 Principle of the assay procedure. (A) The allergen of interest, covalently coupled to ImmunoCAP, reacts with the specific IgE in the patient sample. (B) After washing away nonspecific IgE, enzyme-labeled antibodies against IgE are added to form a complex. (C) After incubation, unbound enzyme–anti-IgE complex is washed away and the bound complex is then incubated with a developing agent. (D) After stopping the reaction, the fluorescence of the eluate is measured. The higher the fluorescence, the more specific IgE is present in the sample. (See color insert.)

12.3.6.4. Noncompetitive Heterogeneous Immunometric Assay Format with the Anti-IgE on Solid Phase. This assay is essentially the reverse of the modified RAST. Here, the anti-IgE antibody is bound to the solid phase and IgE is captured from the sample; finally, drug-specific antibodies are detected by the addition of the labeled drug. This assay format only detects high-affinity antibodies and it is rarely used now due to significant drawbacks. Since the total serum IgE amount varies among humans, and all IgE must be captured from the test specimen, the assay result is variable. In addition, if the concentration of the specific IgE antibody is low in comparison with the total serum IgE, there is a low probability of detecting drug-specific IgE.

12.3.7. A Proposed Assay for Determining Human Anti-Drug IgE

The most frequently used assay format for quantifying specific IgE (sIgE) is probably the noncompetitive heterogeneous immunometric assay, in principle based on the classic radioallergosorbent test, RAST [31]. However, since in most cases no positive control IgE exists at the time of assay development, certain modifications must be made to the basic assay setup. The strategy for assay setup is based on the assumption that, if the biological is allergenic, an IgG immune response will be induced before or at the same time as an IgE

response. Thus, the recommended initial step is to set up a human IgG anti-drug assay. This should be less challenging since circulating levels of IgG are a thousandfold higher than those of IgE and responses are thus easier to detect. Furthermore, guidelines for assays used for the detection of IgG antibodies are available [25–28]. Once the IgG assay has been validated and used to identify IgG-positive subjects, the IgG anti-drug positive serum can be utilized to evaluate the solid-phase component of the IgE assay. The other component of the IgE assay, the labeled anti-human IgE detection antibody, can be quality controlled separately by fulfilling the criteria of exclusive reactivity to human IgE and nondetectable cross-reactivity to other Ig isoforms at physiological concentrations.

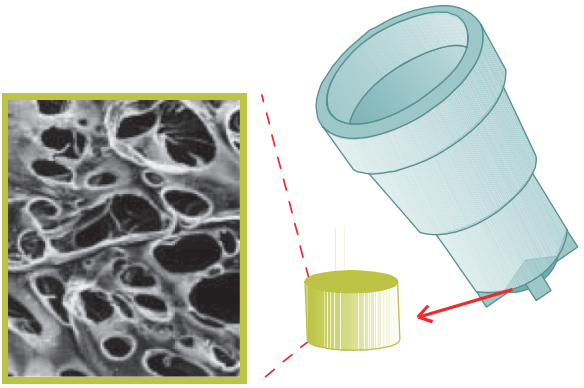
An example of a research assay developed for detecting sIgE that can be extended into a quantitative IgE assay is one developed by Chung et al. [4] to detect IgE directed against cetuximab. It was based on a research tool called ImmunoCAP. In short, cetuximab was biotinylated and added to streptavidin-coated ImmunoCAP before serum was added. Bound IgE antibodies were detected with secondary enzyme-labeled anti-IgE antibodies (Fig. 12.1).

12.3.8. Prerequisites for a Sensitive and Specific Noncompetitive Heterogeneous Immunometric Assay That Permits Quantitative Measurements

There are some prerequisites that must be met when setting up a noncompetitive heterogeneous immunometric IgE assay: the use of an excess amount of allergen, well-defined calibrators, linearity and parallelism for different concentrations, and high assay precision [31–33]. These prerequisites will be discussed further herein.

12.3.8.1. Allergen Must Be Well Characterized and Present in Excess. If allergen bound to the solid phase is present in excess, the assay will be independent of antibody affinity and the quantification will be accurate even when the affinity differs between various specific antibodies. This is shown when applying the law of mass action to a solid-phase heterogeneous assay: when the value of allergen multiplied by the equilibrium constant is greater than 10, the assay is independent of antibody affinity [34]. If the reaction is not affinity-independent the dilution curves of samples and calibrators are not parallel and the results will not be interpretable [35]. Using a solid phase with high binding capacity that enables the presence of an excess amount of antigen thus leads to a robust assay. This method is used in some commercially available systems such as ImmunoCAP (from Phadia), in which an immunosorbent specifically designed for high and reproducible binding capacity is used (Fig. 12.2).

It is also of great importance to verify the reproducibility of different batches of the drug-containing reagent to assure that the assay is specific. Since the drug should be well characterized when entering clinical development, this



L. Sevéus & A. Sandell, 1992

Fig. 12.2 A solid phase with high binding capacity enabling the presence of an excess amount of antigen. (See color insert.)

should be less cumbersome than when analyzing more complex antigens. Qualification could be achieved by using different approaches, for example, competitive binding.

12.3.8.2. Calibrator and Detection Antibodies Must Be Well Defined and Traceable to WHO Standard. Quantitative determination of IgE is possible when using a calibrator that is directly traceable to an internationally recognized standard, the WHO International Reference Preparation for Human IgE 75/102 [34, 36]. Furthermore, anti-human IgE antibodies used in the assay should have an exclusive reactivity to human IgE and nondetectable cross-reactivity to other Ig isoforms at physiological concentrations. This could conveniently be addressed by using a calibrator that is directly traceable to WHO International Reference Preparation for Human IgE 75/102.

12.3.8.3. Assay Quality and Validation. Linearity and parallelism between calibrators and sample dilutions is a second prerequisite for an assay to be acceptable. Criteria for linearity and parallelism of dilution curves and calibrators over the measuring range must be met to validate the IgE assay. In addition, the assay needs high precision; a minimal performance target of 15% coefficient of variation has been proposed by the Clinical and Laboratory Standards Institute (CLSI), which also recommends quality control procedures [37].

In addition, to be acceptable as a test for clinically relevant situations the assay must show high specificity and sensitivity. **Sensitivity** is defined as the proportion of test-positive subjects related to the total number of subjects showing clinical symptoms, whereas **specificity** is defined as the proportion of test-negative subjects in relation to subjects not showing clinical symptoms. Values for specificity and sensitivity of approximately 90% have

been demonstrated for IgE assays. Given the severity of possible anaphylactic reactions to biopharmaceuticals, care should be taken when interpreting values for IgE below the threshold set for judging the test result as positive since even low anti-drug IgE could indicate that the subject is in the process of developing an allergic response.

12.3.9. Assay Interference

Several factors that could interfere with the quantification of the anti-drug IgE must be addressed during the setup or use of the assay. Such factors include high concentration of investigative drug in samples, high IgG concentration, low ratio of sIgE to total IgE, and pre-existing antibodies. All of these factors will be discussed in the following subsections.

12.3.9.1. Interference by High Concentrations of Biopharmaceutical Drug in Samples. High levels of biopharmaceutical drug present at the time of specimen collection will interfere with the assay. The drug in the sample will compete with immobilized drug molecules in binding the drug-specific IgE present in the subject, thus preventing detection of sIgE in the assay. If the biological *in vivo* half-life of the drug is known, this interference can in some cases be circumvented by collecting study subject blood at a time point when the circulating free drug levels are known to be as low as possible.

12.3.9.2. Assay Interference by IgG, High Total IgE, or Pre-existing Specific IgE Antibodies in Specimens. Immunoglobulin G directed against the biological drug may interfere with the assay, since IgE is present in very much lower concentrations than IgG; non-IgE antibodies binding to the biopharmaceutical drug can reduce the binding of the drug-specific IgE antibody. This reduces the ability of the IgE assay to detect drug-specific IgE antibodies. This potential issue can be solved by having an excess amount of allergen present in the assay (as with ImmunoCAP).

In addition, nonspecific binding due to a low ratio of specific IgE to total IgE could interfere with assay performance. If the specific IgE directed against the biopharmaceutical is present only in very low concentrations, there is an increased risk of nonspecific binding and reduced sensitivity of the assay. This could be solved by confirming any positive IgE anti-drug results with a soluble drug competitive inhibition step.

Another concern related to antibodies present in the specimen is pre-existing specific IgE antibodies. In some cases antibodies to biopharmaceuticals are detected in subjects prior to administration of the drug. If these are of an IgG isotype, they may interfere with the anti-drug IgE assay as discussed previously and could be addressed accordingly. However, if the pre-existing antibodies are of IgE isoform as described for cetuximab [4], they may pose clinical consequences and should be analyzed in pretreatment samples, which should be taken from all subjects included in the trials.

12.4. USEFULNESS OF QUANTITATIVE IGE IN RISK MANAGEMENT

12.4.1. Proactivity Is Possible when an Adverse Reaction against a Biological Has Been Confirmed

The incidence of serious adverse drug reactions (ADRs) is high. One metastudy found that the incidence was 6.7% of hospitalized patients [38], while another study found that ADRs account for approximately 5% of all hospital admissions [39]. Adverse drug reactions can be classified as either predictable or unpredictable. Allergic reactions belong to the latter group, whereas toxicity, for example, is predictable and usually dose-dependent. Considering the possibly severe consequences of, for example, an anaphylactic reaction, it would be valuable to be able to predict the risk of allergic reactions with a reliable diagnostic test. Diagnostic tools to detect hypersensitivity to drugs are, however, limited for several reasons [40]. The reaction mechanism may, for instance, be unknown regarding the type of reaction. If an adverse drug reaction is suspected to be an immune response, a confirmatory quantitative *in vitro* IgE test should be carried out in order to decide on a plan of action. If the test is positive, and the allergy confirmed, several options exist to manage this. If possible, or if the reaction was severe, the drug should be avoided. If avoidance is not possible, desensitization should be considered if the reaction is mediated by IgE, or graded challenge if it is non-IgE-mediated. Prophylactic measures, such as a tolerance-induction procedure [41] before administration could also be considered. Taken together, the value of a reliable *in vitro* test is high in determining how to address a suspected anti-drug allergic reaction.

12.4.2. Risk-Management Plan Should Include Immunogenicity

Regulatory authorities state that applicants aiming toward marketing a new drug should present a risk-management plan in which, among other issues, immunogenicity should be addressed and the risk of immune responses during clinical development and post marketing should be considered [42].

12.4.3. Assessment of Allergenicity to Assure Safety and Efficacy in Clinical Trials of Biopharmaceuticals

A crucial part of the clinical development process is to demonstrate the clinical safety of a new drug; this includes determining the drug's potential immunogenicity, where specific care should be taken as to its potential to induce IgE-mediated allergic reactions. Although this chapter focuses specifically on the setup and use of an assay to determine drug-specific human IgE, the importance of clinically evaluating subjects entering a clinical trial should, however, not be underestimated. This evaluation begins with collecting a clinical history from the study subjects by using a questionnaire complemented

with a physical examination, to provide objective estimates of symptoms related to those organs where symptoms of allergic disorders are usually observed. Once a suspected allergic reaction to the drug has been supported by physical examination, it should be verified by *in vivo* or *in vitro* diagnostic methods. *In vivo* tests include the skin prick test (SPT) and provocation tests. Both have drawbacks when used in evaluating allergic reactions to biopharmaceuticals. Skin testing methods have variability associated with techniques and scoring methods [43], in addition to the fact that the stability of the drug used in the SPT could influence the score. *In vivo* provocation tests are suitable mainly for testing allergenicity against drugs that are to be either inhaled or ingested. Since biopharmaceuticals are usually administered via injection these methods are less attractive for evaluating their immunogenicity.

In vitro determination of immunogenicity is often preferred over *in vivo* methods when evaluating the safety of a new biopharmaceutical drug, since patient safety is always a concern when performing an *in vivo* evaluation of a new substance [44].

It is important to note that positive detection of anti-drug IgE in an *in vitro* assay supports the notion that an adverse reaction in a subject is indeed an IgE-mediated reaction. *In vitro* methods also have the advantages of being easier to standardize, having lower coefficients of variation, and being safer clinically than *in vivo* methods. *In vitro* results should also be related to clinical data when possible.

12.4.4. Quantification of Specific IgE for Predicting the Risk of Severe Reactions

It has been shown that an increased concentration of IgE antibodies leads to an increased probability of clinical signs. The quantitative levels of specific IgE can therefore be used to estimate the risk of an allergic reaction, as shown in the case of food allergy [45].The potential value of determining specific IgE levels for predicting the risk of severe reactions to a drug could be illustrated by similar studies where it was shown that cutoff values for sIgE for hen's egg could predict the risk of allergic reactions. Moreover, the *in vitro* test was sufficiently predictive that it could replace oral food challenges that are both time-consuming and potentially dangerous to patients' health [46]. Another study demonstrated that oral food challenge to evaluate cow's milk allergy could be avoided if SPT followed by a quantitative IgE determination were positive [47]. Taken together, a similar regimen could be used to predict and avoid allergic reactions against biopharmaceuticals.

12.4.5. Examples of Benefit of Using IgE Determination to Predict Drug Allergy against Biologicals

The risk of causing antibody formation is greater when using biopharmaceuticals compared with low-molecular-weight drugs [48]. The formation of anti-

drug neutralizing antibodies or allergy and even anaphylaxis are of clinical concern affecting efficacy and safety [49].

Using quantitative determination of human anti-drug-IgE antibodies in clinical trial subjects or patients could decrease the risk of anaphylactic reactions. A recent study of cetuximab-induced anaphylaxis demonstrated the usefulness of a quantitative assay to determine human anti-drug IgE in subjects in a clinical study of the biopharmaceutical drug [4]. In the 76 subjects who were treated with cetuximab, 25 had a hypersensitivity reaction attributed to IgE antibodies against the drug. Surprisingly, IgE antibodies were detected even in pretreatment serum in a majority of the subjects who had to discontinue the treatment. One of the 17 subjects had very low levels of sIgE, illustrating the fact that even modest increases in IgE imply a risk of allergic reactions. Importantly, the high sensitivity and specificity of a positive result in the sIgE assay for a severe hypersensitivity reaction shows that the test would be very useful to identify subjects who are at risk of a severe allergenic ADR. Altogether, this demonstrates that a quantitative IgE assay can be used to identify and possibly exclude patients with a pre-existing sensitivity against the biopharmaceutical drug in order to avoid anaphylactic reactions.

Similarly, it was shown that most reactions against the anti-inflammatory chimeric monoclonal antibody infliximab could be predicted by the presence of IgE antibodies directed against infliximab. This could potentially be used to improve the safety profile for this therapeutic monoclonal antibody [10, 50]. Severe anaphylactic reactions have also been seen when the recombinant monoclonal antibodies adaline and omalizumab have been used in clinical studies [16], although the incidences were lower than in the cetuximab study [4].

12.4.6. Tolerance Induction of Patients with Hypersensitivity Reactions toward a Drug

In cases where a drug is crucial for treating a severe disease, discontinuation is not an option when hypersensitivity to the drug is observed. In those cases, tolerance induction to the allergenic drug might be an alternative route. This was demonstrated when HIV patients showed hypersensitivity reactions to the reverse transcriptase inhibitor nevirapine. Here, a tolerance-induction procedure was successful in several patients who could then continue the treatment [40]. A similar tolerance-induction regimen was conducted for trimethoprim/sulfamethoxazole [51]. This illustrates that even when allergy against a drug has been confirmed by quantitative IgE determination, steps such as tolerance induction can be taken to continue an important treatment. Moreover, the progress of tolerance induction can be monitored by the quantitative determination of IgG, IgG4, and IgE [52–54].

12.5. ALLERGEN LOAD AND IMPACT ON SYMPTOMS

It is well known that overall allergen load can increase the risk of clinical manifestation of novel allergic sensitivities for a subject. Once allergy presents, similarity between allergens frequently causes cross-reactivity patterns, for example, stone fruits–birch pollen allergy [55]. Here, oral allergy symptoms to fruits increase during the pollen season since the total pollen load is increased [56]. This may also be important in sensitization against biopharmaceuticals where a medical history of anaphylactic reactions could be a sign that patients have a higher risk of developing serious allergic reactions against the biological drug. In addition, preformed antibodies against similar allergens as detected by determination of sIgE could be used to identify subjects at risk of allergic reactions [4].

12.6. SUBCLINICAL IGE LEVELS

Quantitative determination of allergen-specific IgE is important in diagnosing allergies. Studies have shown that evaluation of the medical history and a physical examination together give an accuracy of less than 50% in the diagnosis of allergic sensitization, and *in vitro* determination of specific IgE levels must be used to complement these determinations [57]. The amount of specific IgE correlates to the probability of reacting clinically to an allergen [55]. However, the detection of low levels of sIgE can be important in predicting

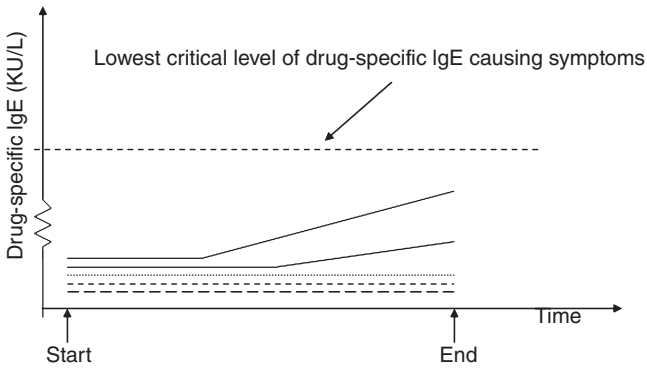


Fig. 12.3 Increasing levels of drug-specific IgE imply a risk of hypersensitivity reactions prior to clinical symptoms. The concentration of specific IgE is depicted on the *y*-axis, and the start and end times of the treatment or clinical trial are depicted on the *x*-axis. Detection of a clinical sign is indicated in the area above the dotted horizontal line. Patients with increasing concentrations of drug-specific IgE (black lines) have a higher risk of a hypersensitivity reaction even if no clinical sign has yet been detected, compared with patients with a stable low level of specific IgE (dotted lines). The graph is intended to be hypothetical and not absolute.

the development of allergic reactions, as demonstrated for predicting allergy in children by detecting low levels of sIgE earlier in life. Since existing IgE detection systems such as ImmunoCAP are very sensitive, sIgE levels as low as less than 0.2 ng/mL can be detected before anaphylactic reactions occur, as described for infliximab [10] (Fig. 12.3).

ACKNOWLEDGMENT

We thank Dr. Mary-Rose Hoja and Dr. Hjalmar Gullberg for linguistic revision of the manuscript.

REFERENCES

1. Pichler, W.J. (2006). Adverse side-effects to biological agents. *Allergy*, *61*, 912–920.
2. Hamilton, R.G. (2008). Human immunoglobulins. In *Handbook of Human Immunology*, 2nd edition. O'Gorman MRG, Donnenberg AD, eds. CRC Press, Boca Raton, FL, pp. 63–106.
3. Chung, C.H. (2008). Managing premedications and the risk for reactions to infusional monoclonal antibody therapy. *Oncologist*, *13*, 725–732.
4. Chung, C.H., Mirakhur, B., Chan, E., Le, Q.T., Berlin, J., Morse, M., et al. (2008). Cetuximab-induced anaphylaxis and IgE specific for galactose-α-1,3-galactose. *N Engl J Med*, *358*, 1109–1117.
5. Johansson, S.G.O., Bieber, T., Dahl, R., Friedmann, P.S., Lanier, B.Q., Lockey, R.F., et al. (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol*, *113*, 832–836.
6. Simons, F.E. (2009). Anaphylaxis: recent advances in assessment and treatment. *J Allergy Clin Immunol*, *124*, 625–636. (Review.)
7. Paganelli, R., Ansotegui, I.J., Sastre, J., Lange, C.E., Roovers, M.H.W.M., de Groot, H. (1998). Specific IgE antibodies in the diagnosis of atopic disease. Clinical evaluation of a new in vitro test system, UniCAP, in six European allergy clinics. *Allergy*, *53*, 763–768.
8. Commins, S.P., Platts-Mills, T.A.E. (2009). Anaphylaxis syndromes related to a new mammalian cross-reactive carbohydrate determinant. *J Allergy Clin Immunol*, *124*, 652–657.
9. Han, P.D., Cohen, R.D. (2004). Managing immunogenic responses to infliximab: treatment implications for patients with Crohn's disease. *Drugs*, *64*, 1767–1777.
10. Vultaggio, A., Matucci, A., Nencini, F., Pratesi, S., Parronchi, P., Rossi, O., Romagnani, S., Maggi, E. (2010). Anti-infliximab IgE and non-IgE antibodies and induction of infusion-related severe anaphylactic reactions. *Allergy*, *65*, 657–61.
11. Abbot Laboratories. (2004). Dear Healthcare Professional Letter November 2004. Available at http://www.fda.gov/downloads/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/UCM166914.pdf (Accessed 2010 May 5.)

12. Paltiel, M., Gober, L.M., Deng, A., Mikdashi, J., Alexeeva, I., Saini, S.S., et al. (2008). Immediate type I hypersensitivity response implicated in worsening injection site reactions to adalimumab. *Arch Dermatol*, *144*, 1190–1194.
13. Dore, R.K., Mathews, S., Schechtman, J., Surbeck, W., Mandel, D., Patel, A., Zhou, L., Peloso, P. (2007). The immunogenicity, safety, and efficacy of etanercept liquid administered once weekly in patients with rheumatoid arthritis. *Clin Exp Rheumatol*, *25*, 40–46.
14. Benucci, M., Manfredi, M., Demoly, P., Campi, P. (2008). Injection site reactions to TNF-α blocking agents with positive skin tests. *Allergy*, *63*, 138–139.
15. Cox, L., Platts-Mills, T.A.E., Finegold, I., Schwartz, L.B., Simons, F.E.R., Wallace, D.V. (2007). American Academy of Allergy, Asthma & Immunology/American College of Allergy, Asthma and Immunology Joint Task Force Report on omalizumab-associated anaphylaxis. *J Allergy Clin Immunol*, *120*, 1373–1377.
16. Limb, S.L., Starke, P.R., Lee, C.E., Chowdhury, B.A. (2007). Delayed onset and protracted progression of anaphylaxis after omalizumab administration in patients with asthma. *J Allergy Clin Immunol*, *120*, 1378–1381. [Review. Erratum in: *J Allergy Clin Immunol* (2008) Jan,*121* (1), 178.]
17. Food and Drug Administration (FDA). (2007). Healthcare professional information. Available at http://www.fda.gov/Drugs/DrugSafety/PostmarketDrugSafetyInformationforPatientsandProviders/ucm126456.htm (Accessed 2010 May 5.)
18. Price, K.S., Hamilton, R.G. (2007). Anaphylactoid reactions in two patients after omalizumab administration after successful long-term therapy. *Allergy Asthma Proc*, *28*, 313–319.
19. Roche Pharmaceuticals. (2003). Dear Healthcare Professional Letter Aug 2003. Available at http://www.fda.gov/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/ucm153212.htm (Accessed 2010 May 5.)
20. Baudouin, V., Crusiaux, A., Haddad, E., Schandene, L., Goldman, M., Loirat, C., Abramowicz, D. (2003) Anaphylactic shock caused by immunoglobulin E sensitization after retreatment with the chimeric anti-interleukin-2 receptor monoclonal antibody basiliximab. *Transplantation*, Aug 15, *76* (3), 459–463.
21. Georgitis, J.W., Browning, M.C., Steiner, D., Lorentz, W.B. (1991). Anaphylaxis and desensitization to the murine monoclonal antibody used for renal graft rejection. *Ann Allergy*, *66*, 343–347.
22. Abramowicz, D., Crusiaux, A., Niaudet, P., Kreis, H., Chatenoud, L., Goldman, M. (1996). The IgE humoral response in OKT3-treated patients: incidence and fine specificity. *Transplantation*, *61*, 577–581.
23. Munoz-Cano, R., Carnéz, J., Sanches-Lopez, J., Saiz, A., Bartra, J., Lopez-Matas, M.A., Picado, C., Valero, A. (2010). Biological agents: new drugs, old problems. *J Allergy Clin Immunol*, *126*, 394–395.
24. Kaplan, S.L., Underwood, L.E., August, G.P., et al. (1986). Clinical studies with recombinant-DNA-derived methionyl human growth hormone in growth hormone deficient children. *Lancet*, *1*, 697–700.
25. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T., Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, Dec 15, *48* (5), 1267–1281. (Review.)

26. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, Apr 20, *333* (1–2), 1–9. (Review.)

27. Mire-Sluis, A,R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, Jun, *289* (1–2), 1–16.

28. Chamberlain, P., Mire-Sluis, A.R. (2003). An overview of scientific and regulatory issues for the immunogenicity of biological products. *Dev Biol* (Basel) *112*, 3–11. (Review.)

29. Fu, Q., Garnham, C.P., Elliott, S.T., Bovenkamp, D.E., Van Eyk, J.E. (2005). A robust, streamlined, and reproducible method for proteomic analysis of serum by delipidation, albumin and IgG depletion, and two-dimensional gel electrophoresis. *Proteomics*, Jul, *5* (10), 2656–2664.

30. Wide, L., Bennich, H., Johansson, S.G. (1967). Diagnosis of allergy by an in-vitro test for allergen antibodies. *Lancet*, Nov 25, *2* (7526), 1105–1107.

31. Yunginger, J.W., Ahlstedt, S., Eggleston, P.A., Homburger, H.A., Nelson, H.S., Ownby, D.R., Platts-Mills, T.A., Sampson, H.A., Sicherer, S.H., Weinstein, A.M., Williams, P.B., Wood, R.A., Zeiger, R.S. (2000). Quantitative IgE antibody assays in allergic diseases. *J Allergy Clin Immunol*, Jun, *105* (6 Pt. 1), 1077–1084. (Review.)

32. Yman, L. (2001). Standardization of in vitro methods. *Allergy*, *56* (Suppl. 67), 70–74. (Review.)

33. Williams, P.B., Barnes, J.H., Szeinbach, S.L., Sullivan, T.J. (2000). Analytic precision and accuracy of commercial immunoassays for specific IgE: establishing a standard. *J Allergy Clin Immunol*, Jun, *105* (6 Pt. 1), 1221–1230.

34. Yman, L. (2000). Allergy. In *The Immunoassay Handbook*, 2nd edition. Wild D, ed. Tradespools, Frome, UK.

35. Lehtonen, O.P., Eerola, E. (1982). The effect of different antibody affinities on ELISA absorbance and titer. *J Immunol Methods*, Oct 29, *54* (2), 233–240.

36. Yman, L. (1991). Standardization of IgE antibody assays. *J Int Fed Clin Chem*, Dec, *3* (5), 198–203.

37. Public Health Service. (1998). Review criteria for the assessment of allergen-specific immunoglobulin E (IgE) in vitro diagnostic devices using immunological methods. Public Health Service, Division of Clinical Laboratory Devices, Office of Device Evaluation, Washington, DC, P 1–18. Clinical laboratory improvement amendment of 1988: final rule. *Federal Regulations* (1992) *57*, 7002–7288; *42* Code of Federal Regulations, Part 405.

38. Lazarou, J., Pomeranz, B.H., Corey, P.N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA*, Apr 15, *279* (15), 1200–1205.

39. Gruchalla, R.S. (2001). Drug metabolism, danger signals, and drug-induced hypersensitivity. *J Allergy Clin Immunol*, Oct, *108* (4) 475–488. (Review.)

40. Gruchalla, R. (2000). Understanding drug allergies. *J Allergy Clin Immunol*, Jun, *105* (6 Pt. 2), S637–S644. (Review.)

41. Demoly, P., Messaad, D., Fabre, J., Reynes, J., Bousquet, J. (1999). Nevirapine-induced cutaneous hypersensitivity reactions and successful tolerance induction. *J Allergy Clin Immunol*, Aug, *104* (2 Pt. 1), 504–505.

42. European Medicines Agency (EMEA). (2005). CHMP guideline on risk management systems for medicinal products for human use, EMEA/CHMP/96268/2005.

43. Nopp, A., Johansson, S.G., Ankerst, J., Bylin, G., Cardell, L.O., Grönneberg, R., Irander, K., Palmqvist, M., Oman, H. (2006). Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy*, Mar, *61* (3), 298–302.

44. Riezzo, I., Bello, S., Neri, M., Turillazzi, E., Fineschi, V. (2010). Ceftriaxone intradermal test-related fatal anaphylactic shock: a medico-legal nightmare. *Allergy*, Jan, *65* (1), 130–131.

45. Söderström, L., Kober, A., Ahlstedt, S., de Groot, H., Lange, C.E., Paganelli, R., Roovers, M.H., Sastre, J. (2003). A further evaluation of the clinical use of specific IgE antibody testing in allergic diseases. *Allergy*, Sep, *58* (9), 921–928.

46. Celik-Bilgili, S., Mehl, A., Verstege, A., Staden, U., Nocon, M., Beyer, K., Niggemann, B. (2005). The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. *Clin Exp Allergy*, Mar, *35* (3), 268–273.

47. García-Ara, C., Boyano-Martínez, T., Díaz-Pena, J.M., Martín-Muñoz, F., Reche-Frutos, M., Martín-Esteban, M. (2001). Specific IgE levels in the diagnosis of immediate hypersensitivity to cows' milk protein in the infant. *J Allergy Clin Immunol*, Jan, *107* (1), 185–190.

48. Purcell, R.T., Lockey, R.F. (2008). Immunologic responses to therapeutic biologic agents. *J Investig Allergol Clin Immunol*, *18* (5), 335–342. (Review.)

49. Tamilvanan, S., Raja, N.L., Sa, B., Basu, S.K. (2010). Clinical concerns of immunogenicity produced at cellular levels by biopharmaceuticals following their parenteral administration into human body. *J Drug Target*, Mar 1 [Epub ahead of print]. PubMed PMID: 20192653

50. Baert, F., Noman, M., Vermeire, S., Van Assche, G., D' Haens, G., Carbonez, A., Rutgeerts, P. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med*, Feb 13, *348* (7), 601–608,

51. Absar, N., Daneshvar, H., Beall, G. (1994). Desensitization to trimethoprim/sulfamethoxazole in HIV-infected patients. *J Allergy Clin Immunol*, Jun, *93* (6), 1001–1005.

52. Akdis, M., Akdis, C.A. (2007). Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol*, Apr, *119* (4), 780–791. (Review.)

53. Mothes, N., Heinzkill, M., Drachenberg, K.J., Sperr, W.R., Krauth, M.T., Majlesi, Y., Semper, H., Valent, P., Niederberger, V., Kraft, D., Valenta, R. (2003). Allergen-specific immunotherapy with a monophosphoryl lipid A–adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy*, Sep, *33* (9), 1198–1208.

54. Valenta, R., Twaroch, T., Swoboda, I. (2007). Component-resolved diagnosis to optimize allergen-specific immunotherapy in the Mediterranean area. *J Investig Allergol Clin Immunol*, *17* (Suppl. 1), 36–40. (Review.)

55. Ahlstedt, S., Murray, C.S. (2006). In vitro diagnosis of allergy: how to interpret IgE antibody results in clinical practice. *Prim Care Respir J*, Aug, *15* (4), 228–236. [Epub 2006 Jul 12.] (Review.)
56. Weber, R.W. (2003). Patterns of pollen cross-allergenicity. *J Allergy Clin Immunol*, Aug, *112* (2), 229–239; quiz 240. (Review.)
57. Williams, P.B., Ahlstedt, S., Barnes, J.H., Söderström, L., Portnoy, J. (2003). Are our impressions of allergy test performances correct? *Ann Allergy Asthma Immunol*, Jul, *91* (1), 26–33.

PART IV

ASSAY STANDARDIZATION AND VALIDATION

14

STANDARDIZATION AND VALIDATION OF CELL-BASED ASSAYS FOR THE DETECTION OF NEUTRALIZING ANTI-DRUG ANTIBODIES

Deborah Finco-Kent and Amy Grenham

14.1. INTRODUCTION

All protein therapeutics have the potential to generate an immune response that could impact safety or efficacy or both. The most severe consequences of immunogenicity on drug safety have been observed when neutralizing antibodies (NAbs) were formed against the protein therapeutic and cross-reacted with an endogenous protein, inhibiting a critical physiological pathway [1]. For example, administration of pegylated megakaryocyte growth and development factor (MGDF) in healthy volunteers induced NAbs that cross-reacted with thrombopoietin (TPO), resulting in severe thrombocytopenia [2]; similarly, red cell aplasia was observed in patients treated with erythropoietin (EPO) due to the development of NAbs against the administered erythropoietin [3]. In other cases, such as for interferon products, the development of neutralizing anti-drug antibodies (ADAs) primarily impacts drug efficacy [4]. These examples demonstrate the potential impact of neutralizing ADAs and why information regarding the neutralizing capacity of ADAs may provide critical information regarding the safety and efficacy of the protein therapeutic.

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15

STANDARDIZATION OF NEUTRALIZING ANTIBODY UNITAGE BY BIOASSAY DESIGN: CONSTANT ANTIGEN AND CONSTANT ANTIBODY METHODOLOGY

Sidney E. Grossberg, Yoshimi Kawade, and
Leslie D. Grossberg

The standardization of the quantitative measurement of antibodies (NAbs) that neutralize biopharmaceutical effector proteins of high specific activity can be readily achieved by designing bioassays to allow for a common means of expression of results as tenfold reduction units per milliliter (TRU/mL). Standardization by such means eliminates the need for a particular, prescribed set of cells, instrumentation, reagents, and procedure. The standardized design benefits from an established history of successful application to cytokine NAbs as well as a developed theoretical basis. This chapter describes in practical terms bioassay design for the use of both the constant antigen and the constant antibody methods to measuring and reporting antibody neutralization.

15.1. INTRODUCTION

Neutralization by an antibody (Ab) can be defined as its inhibition of the biological effect of an antigen. Many, very different, therapeutic biological

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PART V

STATISTICAL CONSIDERATIONS

17

DILUTIONAL LINEARITY FOR NEUTRALIZING ANTIBODY ASSAYS

David Lansky and Carrie Wager

17.1. INTRODUCTION

There is a growing awareness of the need to assess dilutional linearity in bioassays [1]. Neutralizing antibody (NAb) assays, which are nearly all modified bioassays, routinely proceed without assessing similarity among dose–response curves for specimens. Lack of similarity among specimens, in turn, can manifest as specimen-specific dilution bias. In addition, NAb assays typically have a range of activities that is several-fold wider than the dynamic range of the assay, thereby further narrowing the limits on acceptable bias. These two features make dilutional linearity assessment essential for NAb assays, as it is the only assessment of bias.

The primary goal of a dilutional linearity study for a NAb assay is to experimentally document the effect of sample dilution on the precision and bias of the response measure. The sources of error in measures of NAb assay response can be catagorized into five distinct types: measurement bias, truncation bias, population dilution bias, specimen-specific dilution bias, and other sources of variation. To adequately assess dilutional linearity, considerable care must be taken to measure, account for, or understand each of these sources of error.

Using simulated data, we illustrate that it is not reasonable to expect to demonstrate dilutional linearity of a NAb assay without using a design and analysis that estimates the separate contributions of population and specimen-specific dilution biases.

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PART VI

ADAPTATION OF ANTI-DRUG ANTIBODY ASSAYS TO CHALLENGING CONDITIONS

19

ANTIBODY ASSAYS IN ANIMAL RESEARCH

Vera Brinks, Francesca Gilli, Melody Sauerborn, and Huub Schellekens

19.1. INTRODUCTION

Animal studies are an intrinsic part of immunogenicity research of biopharmaceuticals [1]. The upsurge in novel and follow-on biopharmaceuticals during recent decades has increased the use of animals as a (preclinical) predictor for immunogenicity and a model to study the immunological processes underlying immunogenicity. Animal studies also provide the antisera that can be used to develop and validate the assays, which later on can be fine-tuned when sera from treated patients become available. Crucial for this research are, of course, appropriate animal models [2], but also antibody assays suitable for anti-drug antibody (ADA) detection [3–5]. In addition, antibody assays are important to interpret data from toxicological and pharmacokinetic data from the preclinical studies of protein drugs in animals. This chapter focuses on antibody assays in animal research used to detect, quantify, and assess neutralizing activity of ADAs.

19.2. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

19.2.1. Detection of Antibodies against Recombinant Human Therapeutics in Mouse Serum and Plasma

There is an increasing awareness of the problem of immunogenicity in the development of recombinant human (rh) therapeutics. Preclinical assessment

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20

IMMUNOGENICITY OF MICROBIAL DIGESTIVE ENZYMES FOR ORAL REPLACEMENT THERAPY IN PANCREATIC EXOCRINE INSUFFICIENCY

Claudia Berger and Uwe Niesner

20.1. INTRODUCTION

Pancreatic exocrine insufficiency (PEI) is caused by underlying diseases such as cystic fibrosis or chronic pancreatitis. The pathological mechanisms vary according to the etiology of the underlying disease, but in all cases insufficient digestive enzyme secretion by the pancreas results in maldigestion and malabsorption. Left untreated, this leads to malnutrition and can thereby increase morbidity and mortality. In patients with PEI, pancreatic enzyme replacement therapy facilitates the digestion and subsequent absorption of fats, proteins, and carbohydrates. Microbial digestive enzymes represent an alternative to the established oral enzyme replacement therapy with porcine pancreatic extracts. Currently, only a very limited number of medicinal products containing microbial enzymes are approved for treatment of digestive disorders in single countries; however, several products are in development.

The immunogenicity risk assessment for orally applied microbial digestive enzymes follows the same strategy as that used for parenteral biopharmaceuticals. The probability and consequences of adverse immune responses are based on a number of product-related and host-related (i.e., patient or disease)

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PART VII

NOVEL TECHNOLOGIES FOR THE QUANTIFICATION OF NEUTRALIZING ANTIBODIES

22

A NOVEL ONE-STEP CELL-BASED ASSAY FOR QUANTIFICATION OF NEUTRALIZING ANTIBODIES TO BIOPHARMACEUTICALS

Christophe Lallemand and Michael G. Tovey

A unique one-step cell-based assay platform has been developed for the quantification of neutralizing antibodies (NAbs) to biopharmaceuticals that allows both drug concentration and anti-drug NAbs to be quantified in a single serum sample without the need for serial dilution of the sample, addition of exogenous drug, standard curves, external controls, or other manipulation. Drug activity and anti-drug NAbs are quantified with a high degree of precision and within a few hours using cells transfected with a luciferase reporter gene controlled by a drug-responsive chimeric promoter. The assay cells have also been engineered to express and secrete the drug of interest, the production of which is normalized relative to the expression of a second luciferase reporter gene transcribed from a common inducible promoter. Thus, residual drug levels present in a patient sample are first quantified by determination of firefly luciferase expression, prior to induction of autocrine drug synthesis. Drug synthesis is then induced and NAb activity can be quantified instantaneously from the difference in the expression of the drug-responsive reporter gene in the presence or absence of the NAb-containing sample. Since assay results are normalized relative to the expression of an internal standard, results are independent of cell number, differences in cell viability, or errors in sample dilution, thus affording a high degree of assay precision difficult to obtain using a

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conventional NAb assay. This unique assay platform is ideally suited for high-throughput analysis of samples for routine patient monitoring and is applicable to the quantification of both drug activity and anti-drug NAb levels for a wide range of biopharmaceuticals, facilitating comparison of immunogenicity data between assays, laboratories, and compounds.

22.1. INTRODUCTION

Repeated administration of therapeutic proteins, including recombinant homologues of authentic human proteins such as interferon beta (IFN-β) or erythropoietin (EPO), can elicit an immune response leading to the production of anti-drug antibodies (ADAs). These antibodies may bind to the drug in such a manner that they are largely without effect, or they may alter drug pharmacokinetics, or they may even neutralize the activity of the drug by binding to an epitope in or near the active site or by preventing the drug from binding to a receptor on target cells [1]. Two principal approaches are used to detect and quantify neutralizing antibodies (NAbs) *in vitro*: cell-based assays, in which the ability of the antibody to reduce or abrogate the biological activity of a given amount of drug is determined, and non-cell-based competitive ligand-binding assays, in which the ability of the antibody to inhibit binding of the drug to its target molecule or receptor is assessed [1]. Binding to a receptor does not necessarily induce a biological response, however; although in most cases the ability of an ADA to inhibit binding of the drug to its target molecule, or to a soluble receptor immobilized on a solid surface or to immobilized target cells, correlates with an inhibition of biological activity, antibodies have been described that inhibit biological activity without inhibiting binding of the drug to its receptor. For example, Novick et al. [2] have described monoclonal antibodies that inhibit the biological activity of type I IFNs by inducing hyper–tyrosine phosphorylation of one of the two chains of the type I IFN receptor hetrodimer without inhibiting binding of type I IFNs. Thus, loss of biological activity can only be determined conclusively using a functional assay. Cell-based assays that assess the effects of ADAs on drug activity would therefore appear to be the method of choice for detection and quantification of neutralizing ADAs. Although all cell-based assays are composed of similar components (including a drug-responsive cell line, a means of assessing a drug-induced response and the ability of an antibody to inhibit that response, the drug itself, positive and negative controls, and a suitable serum matrix for performing the assay), the form of the assay can vary considerably, reflecting the diversity of therapeutic proteins currently employed in the clinic. These include enzymes, plasma proteins, hormones, recombinant homologues of cytokines such as IFNs or growth factors such as EPO, monoclonal antibodies, and soluble receptors or other recombinant fusion proteins. Drug-induced responses vary from assessment of an antiviral response in IFN-treated cells to stimulation of cell proliferation in the presence of a growth factor such as

EPO or IL-6, or to induction of apoptosis in the presence of tumor necrosis factor alpha (TNF-α) [3]. Such drug-induced responses are complex events involving the transcriptional activation or repression of numerous genes and are influenced by a number of factors that are difficult to control. Given the need to reduce this complexity and develop assays that enable more direct comparisons of immunogenicity data, cells were transfected with the firefly luciferase reporter gene regulated by a drug-responsive chimeric promoter that allows drug activity to be determined selectively and with a high degree of precision within a few hours [4]. In addition, a single common readily measurable parameter, light emission, can be used to assess responsiveness to a variety of different drugs, thereby facilitating comparisons of immunogenicity between assays, drugs, and laboratories.

Cell-based assays are notoriously difficult to standardize, however, due in part to assay variation resulting from changes in culture conditions or genetic and epigenetic changes that can occur as cells are cultivated continuously in the laboratory. Cells can loose chromosomes and can accumulate polymorphisms as well as changes in DNA methylation and histone acetylation, all of which may contribute to assay variation from one day to another. To minimize such variation, master and working cell banks of assay cells were constituted such that each lot of assay cells is prepared from a frozen ampoule and is in an identical physiological condition even when used at considerable time intervals. To minimize assay variation even further, cells were treated with the antimitotic drug vinblastin, and the division-arrested cells were stored frozen until use. Chemically treated division-arrested cells have the added advantage of being less susceptible than untreated cells to serum-based matrixes that may contain toxic factors that can adversely affect cell physiology [4]. Thus, assay-ready cells can be manufactured under current Good Manufacturing Practices (cGMP) and stored frozen for at least 3 years without loss of drug sensitivity [4]. Although these procedures remove many of the limitations of cell-based assays for the semiquantification of drug activity and the effect of ADAs on this activity, such assays remain nevertheless relatively labor intensive and difficult to automate. Thus, as in the case of conventional cell-based assays, semiquantification of anti-drug NAbs using a reporter-gene assay requires the following: serial dilutions of the serum sample to be tested with and without exogenously added drug; simultaneous titration of the drug; and inclusion of positive and negative controls as well as reference reagents when available [1, 4].

To overcome these limitations a "one-step" assay has been developed that allows the level of drug activity and anti-drug NAbs to be determined in a serum sample simply by the addition of reporter cells without further dilution, addition of exogenous drug, titration of the drug, or inclusion of positive and negative controls [5].

The one-step assay is based on a cell line that has been engineered to express and secrete the drug of interest and to concomitantly express a reporter gene transcribed from the same inducible promoter. The cell also contains a second reporter gene controlled by a drug-responsive chimeric promoter

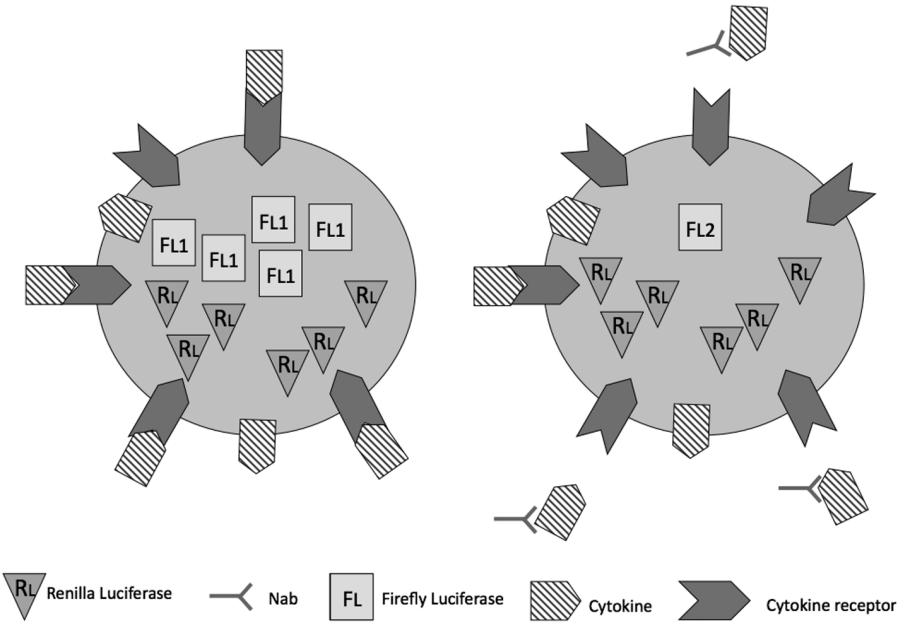

Fig. 22.1 One-step assay for the quantification of NAbs to biopharmaceuticals. One-step assay cells have been engineered to express and secrete the drug of interest and to concomitantly express a reporter gene transcribed from the same inducible promoter. The cell also contains a second reporter gene controlled by a drug-responsive chimeric promoter. Expression of the drug is strictly proportional to expression of the first reporter gene, thereby allowing drug production to be quantified following induction; expression of the second reporter gene allows drug activity to be quantified. The presence of anti-drug NAbs in the immediate environment of the cell will neutralize a quantity of secreted drug, proportional to the neutralizing capacity of the antibody, and thus will prevent drug from binding to and activating its specific cell-surface receptor. This will result in a corresponding reduction in drug-induced activity and hence reduced expression of the drug-responsive reporter gene, the activity of which can be quantified. The degree of reduction in the expression of the drug-responsive reporter gene in the presence or absence of the ADA-containing sample allows the relative neutralizing titer of the sample to be quantified, relative to a given quantity of drug determined from the level of expression the reporter gene transcribed from the same promoter as the drug.

(Fig. 22.1). Expression of the drug is strictly proportional to expression of the first reporter gene, thereby allowing drug production to be quantified with precision following induction, while expression of the second reporter gene allows drug activity to be quantified. The presence of anti-drug NAbs in a sample will neutralize a quantity of drug secreted from the cell, proportional to the neutralizing capacity of the antibody, and thus prevent drug from binding to and activating its specific cell-surface receptor (Fig. 22.1). This will result in a corresponding reduction in drug-induced activity and hence reduced

expression of the drug-responsive reporter gene, the activity of which can be quantified. The degree of reduction in the expression of the drug-responsive reporter gene in the presence or absence of the ADA-containing sample will allow the relative neutralizing titer of the sample to be quantified with respect to a given quantity of drug, determined from the level of expression of the reporter gene transcribed from the same promoter as the drug [5].

Anti-drug NAbs are quantified using two principal approaches: the more common constant antigen method, in which a constant amount of drug is mixed with serial dilutions of test serum [6, 7]; and the more sensitive constant antibody method, in which a set dilution of test serum is mixed with varying concentrations of drug [8]. Residual drug activity is then determined from the median of the maximum and minimum values of the dose–response curve, which is defined as one laboratory unit (LU). The Kawade method of calculation, described in detail in Chapter 15 by Grossberg and colleagues, is frequently used to determine neutralizing titer from the serum dilution that reduces drug activity from 10 to 1 LU/mL or from the drug concentration that reduces the effect of a given dilution of serum to the median of the neutralization curve [6, 7]. For both methods results are expressed in 10-fold reduction units (TRU) per milliliter [6, 7]. The results of anti-drug NAb determinations are at best semiquantitative due to the absence of appropriate standards for anti-drug NAbs.

The one-step assay is based on the same principle as a conventional constant antibody neutralization assay and is applicable to a wide range of biopharmaceuticals. Since results are normalized relative to the expression of an internal standard, and are consequently independent of cell number, this affords a high degree of assay precision. The one-step assay also allows residual drug levels to be quantified in a sample from the expression of the drug-responsive reporter gene prior to induction of autocrine drug synthesis. Drug synthesis is then induced by addition of a promoter-specific inducer such as doxycycline in the case of tetracycline-regulated promoters, and NAb activity is determined in the same sample from the change in expression of the drug-responsive reporter gene in the presence or absence of anti-drug antibodies, without the need for serial dilution of the sample, addition of exogenous drug, or a simultaneous drug dose–response standard curve. Thus, the one-step assay is ideally suited to high-throughput analysis of anti-drug NAbs for routine patient monitoring.

22.2. DEVELOPMENT OF A ONE-STEP ASSAY FOR THE SEMIQUANTIFICATION OF ANTI-HUMAN INTERFERON BETA-1 NEUTRALIZING ANTIBODIES

Human type I IFNs are a family of multifunctional cytokines consisting of 12 IFN-α and single IFN-β, IFN-ω, IFN-ε, and IFN-κ subtypes that constitute an important part of the innate immune response to virus infection [9].

Recombinant homologues of IFN-α2 and IFN-β1 are widely used for the treatment of chronic hepatitis C (HCV) and relapsing–remitting multiple sclerosis (RRMS), respectively [10. 11]. Repeated treatment with recombinant IFN-α2 or IFN-β1 can cause a break in immune tolerance to self-antigens in some patients, resulting in the production of anti-IFN NAbs [12–16]. Such antibodies are considered to be autoantibodies as they cross-react with the corresponding endogenous protein [17]. The frequency of appearance of anti-IFN NAbs appears to be greater in patients treated with IFN-β1 than in patients treated with IFN-α2, for reasons that remain unclear but may include differences in the duration of treatment and differences in glycosylation of the two molecules [14]. There is evidence to suggest that the appearance of anti-IFN-β1 NAbs in RRMS patients is associated with both reduced pharmacodynamics [18] and a reduced clinical response, determined by either magnetic resonance imaging (MRI) or disease progression [16]. The significant lapse of time that can occur between the first appearance of anti-IFN-β1 NAbs and a detectable effect on clinical response would suggest that routine testing of patients for the presence anti-IFN-β1 NAbs would allow patients to be switched to alternative therapy more rapidly.

The presence of anti-IFN-β1 NAbs can be determined either directly *in vivo* or *ex vivo* by the quantification of IFN bioactivity, usually by evaluating induction of IFN-induced genes [19] or encoded proteins [20], as described in Chapter 7 by Deisenhammer, or *in vitro* using a cell-based bioassay [3], as described in Chapter 8 by Meager.

Cell-based assays for the detection and semiquantification of anti-IFN-β1 NAbs are based for the most part on either the ability of IFN-β to inhibit virus replication or on the induction of an IFN-induced protein such as the myxovirus resistance protein A (MxA). Bioassays based on the ability of IFNs to inhibit virus replication are time-consuming, require skilled operators, and frequently display poor reproducibility. Assays based on the induction of an IFN-induced protein such as MxA require the use of IFN-sensitive cell lines or peripheral blood mononuclear cells, and subsequent evaluation of protein levels by enzyme-linked immunosorbent assay (ELISA) or measurement of mRNA levels [20–22]. More recently we and others have described reporter-gene assays for the evaluation of IFN activity and the semiquantification of anti-IFN NAbs [4, 23]. Such assays are based on the establishment of an IFN-sensitive cell line containing a reporter gene such as firefly luciferase under the control of an IFN-responsive chimeric promoter [4]. Such assays offer a highly sensitive and reproducible method for evaluating type I IFN activity and for the semiquantification of anti-IFN NAbs but remain relatively labor intensive, are subject to operator error, and are difficult to automate [4, 23].

To develop a method suitable for high-throughput analysis for routine patient screening, a one-step assay for the detection and semiquantification of anti-IFN-β1 NAbs was developed.

Human type I IFNs bind to a common high-affinity hetrodimeric cell surface receptor composed of the transmembrane polypeptides IFNAR1 and IFNAR2.

IFN binding results in the phosphorylation of the Janus kinases Jak1 and Tyk2, which in turn phosphorylate and activate the latent cytoplasmic signal transducers and activators of transcription (STAT1 and STAT2), leading to the formation of a transcription complex [24], together with IFN regulatory factor 9 (IRF9). Translocation of this complex to the nucleus and interaction with a specific sequence, the interferon sensitive responsive element (ISRE), present in the promoter region of interferon-regulated genes, results in the transcriptional activation of a specific set of genes that encode the effector molecules responsible for mediating the biological activities of the type I IFNs [24].

Human pro-monocytic U937 cells were transfected with the firefly luciferase reporter gene controlled by an interferon-responsive chimeric promoter containing an SV40 minimal promoter and the ISRE from the interferon sensitive gene (ISG) 15 as described previously [4]. These cells were then co-transfected with a vector comprising the coding sequence of human IFN-β1a gene, including its natural signal peptide, to ensure efficient secretion of the drug, together with the coding sequence of *Renilla* luciferase, under the control of a Tet-responsive element (TRE)–*Cytomegalovirus* (CMV) chimeric promoter (Fig. 22.2). The cells were also transfected with a vector encoding the reverse Tet-controlled transactivation protein that confers tetracycline/doxycycline (Tet)-induced gene expression (Fig. 22.2). The *Renilla* luciferase reporter gene and the human IFN-β1a gene were expressed under the control

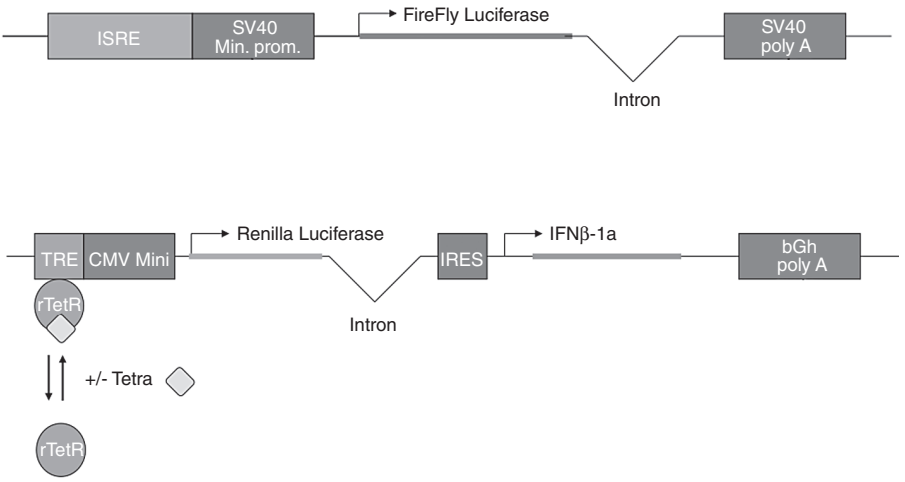

Fig. 22.2 One-step assay for the quantification of NAbs against human IFN-β. Molecular construction: ISRE, interferon sensitive response element; SV40 Min. Prom, SV40 minimal promoter; Intron, intron from the human β-globulin gene; SV40 Poly A, SV40 polyadenylation site; FireFly Luciferase, coding region of the firefly luciferase gene; TRE, tetracycline responsive element; rTetR, reverse tetracycline repressor; Tetra, tetracycline; CMV Mini, CMV minimum promoter; IFNβ-1a, signal peptide and coding region of the human interferon beta-1a gene; IRES, internal ribosomal entry site; Renilla Luciferase, coding region of the *Renilla* luciferase gene.

of the same doxycycline-inducible (Tet-On) CMV promoter in order, on the one hand, to prevent constitutive expression of human type I IFNs from inhibiting cell proliferation and hence preventing cultivation of the transfected cell line, and on the other hand to ensure that IFN production is strictly proportional to *Renilla* luciferase expression. The human IFN-β1a–*Renilla* luciferase reporter gene construction also contains an internal ribosome entry site (IRES) to ensure that the primary RNA transcript is translated into two distinct native proteins (IFN-β1a and *Renilla* luciferase) in order to preserve the tertiary structure of the human IFN-β1a protein and hence its recognition by anti-IFN-β1 antibodies.

Treatment of the IFN-β1a one-step cells with doxycycline results in a dose-dependent increase in the expression of *Renilla* luciferase accompanied by a corresponding increase in IFN-β1a expression and secretion of IFN-β1a into the culture supernatant. Increased expression of IFN-β1a induces a corresponding increase in IFN-responsive firefly luciferase expression some 4 to 6 hours after addition of doxycycline. Firefly luciferase levels then increase progressively up to 12 hours postinduction (Fig. 22.3). To reduce the effective assay time to a minimum, cells were treated for 2 hours with doxycycline, frozen, and then thawed immediately prior to use. Assay-ready IFN-β1a one-step cells can be stored for several months at –80°C without loss of sensitivity.

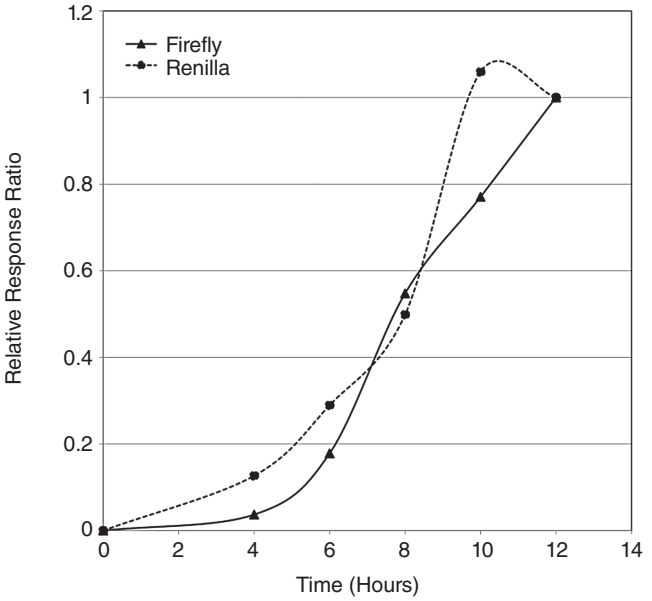

Fig. 22.3 Human IFN-β1 one-step assay cells—kinetics of firefly and *Renilla* luciferase expression. One-step IFN-β1 assay cells were treated with 800 ng/mL of doxycycline; at various times thereafter, firefly and *Renilla* luciferase expression were determined sequentially in the same well using the Dual-Glo luciferase assay system (Promega, Madison, WI).

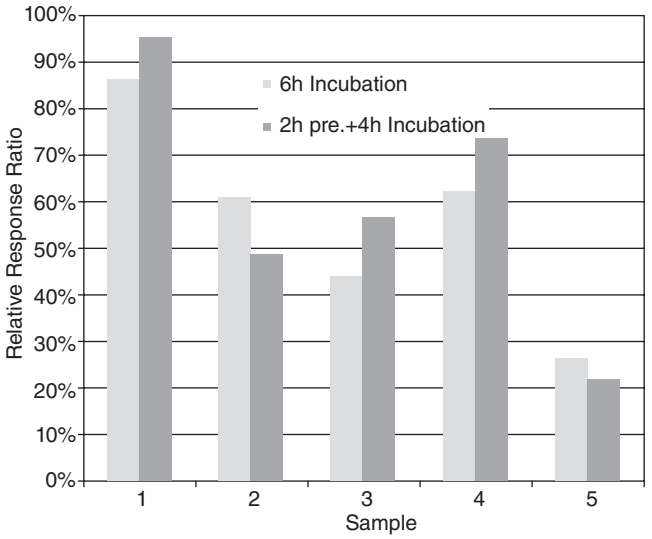

Fig. 22.4 Quantification of NAbs against human IFN-β using the IFN-β1 one-step assay. One-step IFN-β1 assay cells were treated in duplicate with either 800 ng/mL of doxycycline alone or with deoxycycline and a 1:20 dilution of the human serum indicated in the figure for 2 hours and then frozen, thawed, and incubated for a further 4 hours in the presence 800 ng/mL of doxycycline or alternatively incubated continuously together with 800 ng/mL of doxycycline alone for 6 hours or with deoxycycline and a 1:20 dilution of the human serum indicated in the figure. The activities of both firefly and *Renilla* luciferase were then determined sequentially in the same well using the Dual-Glo luciferase assay system (Promega, Madison, WI). The neutralizing activity of the NAb sample was determined from the ratio of the activity of firefly luciferase of the NAb-containing sample, normalized relative to *Renilla* luciferase expression, and the activity of firefly luciferase of the control sample, normalized relative to *Renilla* luciferase expression of the control sample.

Once thawed, cells are mixed with the serum sample to be assayed at a final dilution of 1:10 in culture medium containing an appropriate concentration of doxycycline for 4 hours prior to the addition of lysis buffer and the determination of *Renilla* and firefly luciferase activity (Fig. 22.4). The neutralizing titers of sera from patients with RRMS treated with IFN-β1 were found to be similar whether cells were treated with doxycycline for 2 hours, frozen, thawed, and treated with doxycycline for a further 4 hours or were treated continuously with doxycycline for 6 hours (Fig. 22.4). In agreement with previous results from our laboratory and other laboratories using conventional assays for the detection of neutralizing anti-IFN-β antibodies, anti-IFN NAbs from patients treated with IFN-β1a cross-neutralized NAbs from patients treated with IFN-β1b and vice versa [4, 25]. It has also been reported previously that human polyclonal antibodies directed against human IFN-β do not cross-neutralize human IFN-α, and anti-IFN-α NAbs do not cross-neutralize IFN-β [4].

A high degree of correlation was observed for the anti-IFN-β neutralizing titers of individual human sera obtained from RRMS patients treated with anti-IFN-β1, using IFN-β1a one-step method compared with the results obtained using a conventional constant antibody anti-IFN-β NAb assay when results were expressed in TRU/mL.

The results obtained using the IFN-β1a one-step NAb assay are normalized relative to the expression of an internal standard, *Renilla* luciferase expression, and consequently are not influenced by variations in cell number or errors in sample dilution, thereby affording a degree of assay precision difficult to obtain using a conventional NAb assay.

22.3. DEVELOPMENT OF A ONE-STEP ASSAY FOR THE SEMIQUANTIFICATION OF NEUTRALIZING ANTIBODIES AGAINST TUMOR NECROSIS FACTOR ALPHA ANTAGONISTS

Tumor necrosis factor alpha (TNF-α) antagonists are used widely to treat chronic autoimmune and inflammatory diseases such as Crohn's disease, rheumatoid arthritis (RA), and psoriasis [26–28]. The principal licensed TNF-α antagonists vary considerably in structure and include the partially human chimeric monoclonal antibody infliximab, fully human monoclonal antibodies such as adalimumab and golimumab, pegylated anti-TNF-α Fab2 fragments certolizumab pegol, and the IgG-Fc–TNF p75 receptor fusion protein etanercept [29]. The humoral response to TNF-α antagonists varies markedly, reflecting differences in the structure of individual drugs [30]. For example, infliximab, a mouse–human chimeric monoclonal antibody, elicits a higher frequency of ADA antibodies in patients with RA than adalimumab, a fully human monoclonal antibody [30]. High levels of anti-infliximab NAbs have also been reported in patients with Crohn's disease [31, 32]. Concomitant immunosuppressive therapy has been reported to reduce the antibody response to both adalimumab and infliximab [31, 32]. In contrast, etanercept appears to induce a relatively low incidence of neutralizing antibodies [31]. In patients with Crohn's disease, development of antibodies to infliximab is associated with a higher frequency of infusion reactions and a reduced duration of the observed clinical response [33]. In patients with RA treated with either adalimumab or infliximab, development of ADAs has again been reported to be associated with a reduced clinical response [34].

Tumor necrosis factor alpha antagonists function by binding to TNF-α, thereby preventing TNF-α from binding to its specific cell-surface receptor and activating signal transduction and biological activity [35]. Thus, in order to develop a one-step assay for the detection and semiquantification of the TNF-α antagonist infliximab, human reporter cells carrying a TNF-α-specific cell-surface receptor were stably transfected with a ligand-specific promoter that drives transcription of a luciferase reporter gene. Since TNF-α signals principally by activation of the NFκB pathway, human TNF-α receptor posi-

tive cells were transfected with a chimeric promoter consisting of a 5× tandem repeat of the canonical NFκB recognition site and an SV40 minimal promoter regulating transcription of a second luciferase reporter gene.

Although numerous agents in common with TNF-α signal through the NFκB pathway, a degree of specificity sufficient to allow a TNF-α-specific assay to be developed can be obtained by the transfection of cells that are either receptor negative or insensitive to agents other than TNF-α that also signal through NFκB (Fig. 22.5). Treatment of reporter cells with vinblastin allows assay-ready cells to be stored frozen for prolonged periods without loss of TNF-α sensitivity or the need for cell cultivation, avoiding assay variation associated with cell proliferation. Assays for neutralizing antibodies against infliximab, adalimumab, and etanercept have been developed and the results determined using the Kawade method of calculation. The results of these

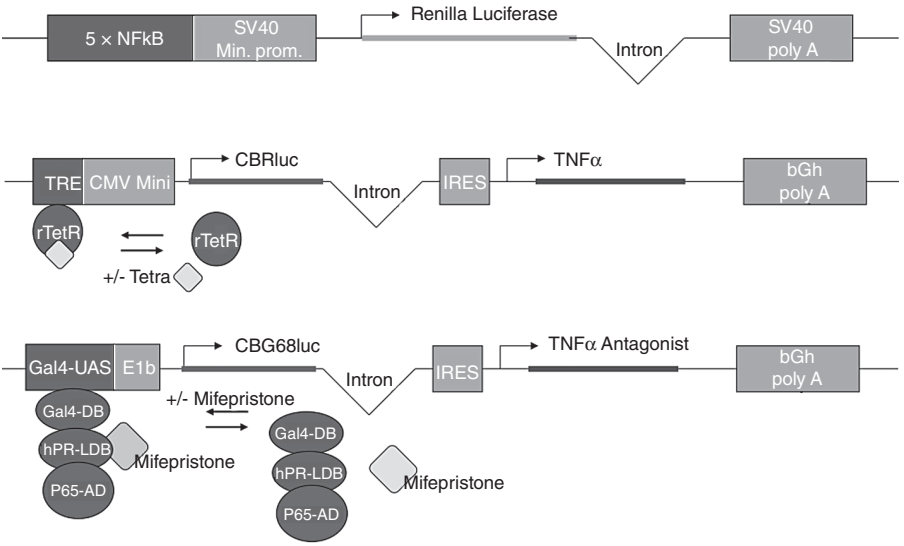

Fig. 22.5 One-step assay for the quantification of NAbs against human TNF-α antagonists. Molecular construction: 5× NFkB, five times tandem repeat of the canonical NFκB recognition sequence; SV40 Min. Prom, SV40 minimal promoter; Renilla Luciferase, coding region of the *Renilla* luciferase gene; Intron, intron from the human β-globulin gene; SV40 Poly A, SV40 polyadenylation site; TRE, tetracycline responsive element; rTetR, reverse tetracycline repressor; Tetra, tetracycline; CMV Mini, cytomegalovirus immediate early minimum promoter; CBRluc, coding region of the *CBR* luciferase gene; TNFα, signal peptide and coding region of the human TNF-α gene; IRES, *Cytomegalovirus* internal ribosomal entry site; HβGl Poly A, polyadenylation site from the human β-globulin gene; GAL4-UAS-E1b, GAL4-UAS and TATA box from the *Adenovirus* E1b minimal promoter; CBG68luc, coding region of the *CBG68* luciferase gene; TNFα Antagonist, coding sequence of the gene(s) encoding the function domain(s) of the TNF-α antagonist; hPR-LDB, truncated human progesterone receptor ligand-binding domain.

studies have shown that circulating levels of infliximab, adalimumab, and etanercept can be detected with ease in patients with rheumatoid arthritis (RA) treated with these drugs. Furthermore, NAbs against infliximab, adalimumab, or etanercept can also be readily detected and quantified with precision using this standardized platform technology.

Development of a one-step assay for the detection and semiquantification of a TNF-α antagonist such as infliximab necessitates transfection of human reporter cells carrying a TNF-α-responsive reporter gene with two additional constructions comprising an expression vector for TNF-α and the hypervariable regions of the TNF-α antagonist infliximab. Each protein is transcribed from an inducible promoter in common with its own specific reporter gene such that the expression of each reporter gene can be measured separately as illustrated in Figure 22.6. Each expression vector contains either the coding sequence of the gene encoding TNF-α or the functional domain(s) of the TNF-α antagonist together with the coding sequence of a strong signal peptide, to ensure efficient secretion of the drug, as well as the coding sequence of the specific luciferase reporter gene, under the control of an inducible promoter and an IRES to ensure that the primary RNA transcript is translated into two distinct native proteins in order to preserve the tertiary structure of the proteins and hence recognition by the respective antibodies. As in the case of IFN-β1, the TNF-α antagonist one-step assay allows both residual drug levels and anti-drug NAbs to be determined sequentially.

22.4. DISCUSSION

Biopharmaceuticals are used increasingly to treat chronic diseases, including various forms of cancer, autoimmune disease, and inflammatory disease, and are responsible for significant advances in the treatment of a number of serious life-threatening and debilitating diseases. Protein-based therapeutics have the potential, however, to elicit an immune response resulting in the production of anti-drug antibodies. Although in some cases appearance of ADAs may be without any apparent untoward effect, ADAs can adversely affect drug pharmacokinetics and may also neutralize the activity of the drug, resulting in a reduced clinical response or treatment failure [13, 16, 30, 33, 34]. ADAs can also induce hypersensitivity or autoimmunity and can be potentially life threatening in the case of the development of NAbs that cross-react with a nonredundant endogenous protein such as EPO or megakaryocyte growth and development factor [36, 37]. The potential of such drugs to elicit an immune response resulting in the production of anti-drug antibodies [38] emphasizes the importance of regularly monitoring patients for the presence of ADAs and anti-drug NAbs in particular, during treatment with biopharmaceuticals. The use of currently available cell-based assays for the detection and semiquantification of anti-drug NAbs is time consuming and relatively expensive; the establishment of widespread routine monitoring of patients for anti-drug

NAbs will necessitate the development of rapid and fully automated standardized methodologies for the quantification of anti-drug antibodies.

A one-step assay platform has been developed that allows both residual drug levels and anti-drug NAbs to be quantified in a sample simply by the addition of reporter cells that carry a drug-responsive reporter gene and are engineered to express and secrete the drug of interest. Thus, residual drug levels and anti-drug NAbs can be quantified rapidly and with a high degree of precision without the need for serial dilution of the sample, addition of exogenous drug, a simultaneous drug dose–response standard curve, or positive and negative controls. One-step assays can be readily automated and are ideally suited to high-throughput analysis of anti-drug NAbs for routine patient monitoring. The one-step assay format is applicable to a wide range of biopharmaceuticals, and one-step assays have been developed for cytokines such as human IFN-α2 and human IFN-β1a and monoclonal antibodies such as the anti-TNF-α antagonist infliximab. The availability of a standardized assay format that can be used to assess the ADA NAb response to a variety of different drugs greatly facilitates comparisons of immunogenicity between assays, drugs, and laboratories.

ACKNOWLEDGMENT

This work was supported in part by a grant from Biomonitor Ltd., including a fellowship to C.L.

REFERENCES

1. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
2. Novick, D., Nabioullin, R.R., Ragsdale, W., McKenna, S., Weiser, W., Garone, L., Burkins, C., Kim, S.H., Rubinstein, M., Tepper, M.A., Arulanandam, A.R. (2000). The neutralization of type I IFN biologic actions by anti-IFNAR-2 monoclonal antibodies is not entirely due to inhibition of Jak–Stat tyrosine phosphorylation. *J Interferon Cytokine Res*, *20*, 971–982.
3. Meager, A. (2006). Measurement of cytokines by bioassays: theory and application. *Methods*, *38*, 237–252.
4. Lallemand, C., Meritet, J.F., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., Lebon, P., Tovey, M.G. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
5. Lallemand, C., Meritet, J.F., Lebon, P., Tovey, M.G. (2010). One-step assay for quantification of neutralizing antibodies to interferon alpha. *J Immunol Methods*, *356*, 18–28.

6. Grossberg, S.E., Kawade, Y., Kohase, M., Yokoyama, H., Finter, N. (2001). The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. *J Interferon Cytokine Res*, *21*, 729–742.
7. Grossberg, S.E., Kawade, Y., Kohase, M., Klein, J.P. (2001). The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. *J Interferon Cytokine Res*, *21*, 743–755.
8. Grossberg, S.E., Kawade, Y., Grossberg, L.D. (2009). The neutralization of interferons by antibody. III. The constant antibody bioassay, a highly sensitive quantitative detector of low antibody levels. *J Interferon Cytokine Res*, *29*, 93–104.
9. Takeuchi, O., Akira, S. (2009). Innate immunity to virus infection. *Immunol Rev*, *227*, 75–86.
10. Paolicelli, D., Direnzo, V., Trojano, M. (2009). Review of interferon beta-1b in the treatment of early and relapsing multiple sclerosis. *Biologics*, *3*, 369–376.
11. McHutchison, J.G., Lawitz, E.J., Shiffman, M.L., Muir, A.J., Galler, G.W., McCone, J., Nyberg, L.M., Lee, W.M., Ghalib, R.H., Schiff, E.R., Galati, J.S., Bacon, B.R., Davis, M.N., Mukhopadhyay, P., Koury, K., Noviello, S., Pedicone, L.D., Brass, C.A., Albrecht, J.K., Sulkowski, M.S. (2009). Peginterferon alfa-2b or alfa-2a with ribavirin for treatment of hepatitis C infection. *N Engl J Med*, *361*, 580–593.
12. van der Eijk, A.A., Vrolijk, J.M., Haagmans, B.L. (2006). Antibodies neutralizing peginterferon alfa during retreatment of hepatitis C. *N Engl J Med*, *354*, 1323–1324.
13. Bertolotto, A. (2009). Implications of neutralising antibodies on therapeutic efficacy. *J Neurol Sci*, *277* (Suppl. 1), S29-S32.
14. Deisenhammer, F. (2009). Neutralizing antibodies to interferon-beta and other immunological treatments for multiple sclerosis: prevalence and impact on outcomes. *CNS Drugs*, *23*, 379–396.
15. Koch-Henriksen, N., Sorensen, P.S., Bendtzen, K., Flachs, E.M. (2009). The clinical effect of neutralizing antibodies against interferon-beta is independent of the type of interferon-beta used for patients with relapsing-remitting multiple sclerosis. *Mult Scler*, *15*, 601–605.
16. Kappos, L., Clanet, M., Sandberg-Wollheim, M., Radue, E.W., Hartung, H.P., Hohlfeld, R., Xu, J., Bennett, D., Sandrock, A., Goelz, S. (2005). Neutralizing antibodies and efficacy of interferon beta-1a: a 4-year controlled study. *Neurology*, *65*, 40–47.
17. Pachner, A.R. (2008). Measurement of binding anti-IFNbeta antibodies in IFNbeta-treated MS patients: lessons for the clinician and basic scientist. *J Neuroimmunol*, *194*, 5–6.
18. Bertolotto, A., Gilli, F., Sala, A., Capobianco, M., Malucchi, S., Milano, E., Melis, F., Marnetto, F., Lindberg, R.L., Bottero, R., Di Sapio, A., Giordana, M.T. (2003). Persistent neutralizing antibodies abolish the interferon beta bioavailability in MS patients. *Neurology*, *60*, 634–639.
19. Pachner, A.R., Narayan, K., Pak, E. (2006). Multiplex analysis of expression of three IFNbeta-induced genes in antibody-positive MS patients. *Neurology*, *66*, 444–446.

20. Deisenhammer, F., Schellekens, H., Bertolotto, A. (2004). Measurement of neutralizing antibodies to interferon beta in patients with multiple sclerosis. *J Neurol*, *251* (Suppl. 2), II31–II39.

21. Moore, M., Meager, A., Wadhwa, M., Burns, C. (2009). Measurement of neutralising antibodies to type I interferons by gene expression assays specific for type 1 interferon-inducible 6–16 mRNA. *J Pharm Biomed Anal*, *49*, 534–539.

22. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., Marnetto, F., Gilli, F. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321*, 19–31.

23. Lam, R., Farrell, R., Aziz, T., Gibbs, E., Giovannoni, G., Grossberg, S., Oger, J. (2008). Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFNbeta in multiple sclerosis patients. *J Immunol Methods*, *336*, 113–118.

24. Schindler, C., Plumlee, C. (2008). Inteferons pen the JAK-STAT pathway. *Semin Cell Dev Biol*, *19*, 311–318.

25. Khan, O.A., Dhib-Jalbut, S.S. (1998). Neutralizing antibodies to interferon beta-1a and interferon beta-1b in MS patients are cross-reactive. *Neurology*, *51*, 1698–1702.

26. Mahadevan, U. (2007). TNF-alpha antagonists: benefits beyond remission. *Rev Gastroenterol Disord*, *7* (Suppl. 1), S13-S19.

27. Bansback, N., Sizto, S., Sun, H., Feldman, S., Willian, M.K., Anis, A. (2009). Efficacy of systemic treatments for moderate to severe plaque psoriasis: systematic review and meta-analysis. *Dermatology*, *219* (3), 209–218.

28. Gibbons, L.J., Hyrich, K.L. (2009). Biologic therapy for rheumatoid arthritis: clinical efficacy and predictors of response. *BioDrugs*, *23*, 111–124.

29. Wallis, S., Guptha, S. (2008). Thromboprophylaxis for patients at high risk of VTE. *Lancet*, *371*, 1911–1912; author reply 1912–1913.

30. Aarden, L., Ruuls, S.R., Wolbink, G. (2008). Immunogenicity of anti-tumor necrosis factor antibodies–toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20*, 431–435.

31. Anderson, P.J. (2005). Tumor necrosis factor inhibitors: clinical implications of their different immunogenicity profiles. *Semin Arthritis Rheum*, *34*, 19–22.

32. Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., Antoni, C., Leeb, B., Elliott, M.J., Woody, J.N., Schaible, T.F., Feldmann, M. (1998). Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum*, *41*, 1552–1563.

33. Baert, F., Noman, M., Vermeire, S., Van Assche, G., D'Haens, G., Carbonez, A., Rutgeerts, P. (2003). Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med*, *348*, 601–608.

34. Radstake, T.R., Svenson, M., Eijsbouts, A.M., van den Hoogen, F.H., Enevold, C., van Riel, P.L., Bendtzen, K. (2008). Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. *Ann Rheum Dis*, *68*, 1739–1745.

35. Kaymakcalan, Z., Sakorafas, P., Bose, S., Scesney, S., Xiong, L., Hanzatian, D.K., Salfeld, J., Sasso, E.H. (2009). Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding to soluble and membrane tumor necrosis factor. *Clin Immunol*, *131*, 308–316.
36. Neumann, T.A., Foote, M. (2000). Megakaryocyte growth and development factor (MGDF): an Mpl ligand and cytokine that regulates thrombopoiesis. *Cytokines Cell Mol Ther*, *6*, 47–56.
37. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.
38. Schellekens, H. (2008). How to predict and prevent the immunogenicity of therapeutic proteins. *Biotechnol Annu Rev*, *14*, 191–202.

21

MEASUREMENT OF BIOLOGICALLY ACTIVE DRUG AS AN APPROACH TO DETECTION OF ANTI-DRUG NEUTRALIZING ANTIBODIES

Yao Zhuang and Shalini Gupta

21.1. INTRODUCTION

Cell-based assays are frequently employed for the detection of anti-drug neutralizing antibodies. Immunoassays that tend to be more sensitive and high throughput in their ability to detect anti-drug antibodies (ADAs) comprise the first tier of assays. Test samples found to be positive in the ADA immunoassay may be subjected to analysis in a cell-based assay to determine if the ADAs have any capability to neutralize or inhibit the biological activity of the drug. Circulating drug may cause interference in ADA detection for both immunoassays and bioassays, especially if the drug is dosed at high concentrations and demonstrates a long half-life. The drug-quantitation-based neutralizing-antibody (DQ-NAb) assay is a recently described approach that is based on the detection of biologically active drug in test samples. This approach is designed to address the acute susceptibility of NAb assays to the presence of circulating drug that hinders NAb detection and utilizes a two-step approach. The first step includes a quantitative determination of the bioactivity of the circulating drug in immunoassay-positive samples. It is expected that if the sample contains clinically effective neutralizing antibodies, the bioactivity of any circulating drug will be impaired. The second step of the DQ-NAb

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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approach assesses the analytical recovery of a known concentration of drug added to the same sample. Any loss or impairment of recovery of the added concentration of drug provides a confirmation that the antibodies detected by the immunoassay are neutralizing in nature. This dual-pronged approach comprises a NAb detection system that provides clinically relevant information relating to the impact of NAbs on drug efficacy and drug safety when high levels of circulating drug pose a high degree of interference in conventional NAb assays.

21.2. BACKGROUND

All protein therapeutics are potentially immunogenic and can cause unwanted immunogenicity and induce the formation of ADAs. Depending on where they bind, ADAs may have different effects on drug activity and can be categorized as neutralizing antibodies (NAbs) or non-neutralizing antibodies, based on their ability or inability to inhibit the biological activity of the drug, respectively. Neutralizing antibodies are those that can inhibit the activity of the drug once bound to them, while non-neutralizing antibodies do not inhibit the therapeutic protein's activity even when they are bound to the drug. Since NAbs directly inhibit drug activity, they have the potential to reduce drug exposure and thereby impact drug efficacy. In addition, if the drug shares similar epitope(s) with an endogenous protein, anti-drug NAbs could also inhibit the activity of the endogenous protein and could potentially inhibit an endogenous pathway essential for a biological function. For these reasons, it is important to assess the neutralizing capability for any anti-drug binding antibodies that are detected in the screening immunoassay [1].

Depending on the drug's mechanism of action, a conventional cell-based assay may utilize a direct or indirect NAb assay format [2] in pooled normal or target subject population serum. Both formats utilize an assay system in which the cells are exposed to a fixed concentration of drug in the selected assay matrix (Fig. 21.1). An assay cut point that represents the threshold response of the cells to the selected concentration of the drug is established using responses observed with individual donor sera and applying appropriate statistical approaches [1, 2]. The effect of the test sample serum on the response of the cells to the drug is evaluated and upon comparing it to the assay cut point, if an inhibition of drug activity is observed, the sample is suspected to contain neutralizing ADAs, which may or may not require further confirmation or characterization.

Monoclonal antibody therapeutics are emerging as useful biotechnology-derived drug products for various diseases. These drugs are usually dosed at high levels and have long half-lives; consequently, antibody sampling time points result in samples containing high levels of circulating drug. The presence of circulating drug in a sample interferes with the NAb assay because the conventional NAb assay is optimized and validated for use with a known fixed

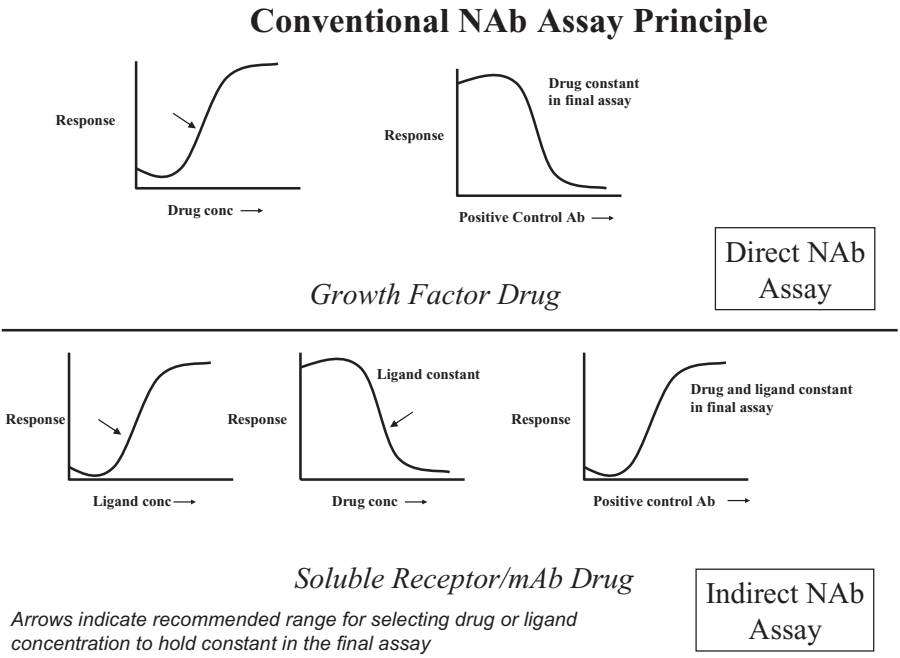

Fig. 21.1 Model depicting the underlying principle of NAb assay design for drug molecules that exhibit agonistic (growth factor) or antagonistic [soluble receptors or monoclonal antibodies (mAbs) that block target ligands] mechanism of action. Arrows indicate the recommended range for selecting drug or ligand concentration to hold constant in the final assay. Direct NAb assays are employed for agonist drugs. In these assays, treating the cells with increased drug concentrations results in proportionally high response values (top panel, left curve), and a neutralizing positive control ADA results in a suppression of the assay response. Indirect NAb assays are utilized for drugs with an antagonistic mechanism of action. These assays require evaluation and selection of the appropriate concentrations of ligand and drug to hold constant in the final assay for NAb determination. A neutralizing positive control ADA typically results in an overcoming of the inhibition of the drug's ability to block the ligand, resulting in an overall increase in assay response.

concentration of the drug. The sensitivity of conventional NAb assays is dependent upon the concentration of drug used in the assay, and efforts are made to select a drug concentration that lies on the linear portion of the drug's dose–response curve. Therefore, the introduction of additional drug from the samples impairs assay sensitivity for NAb detection and results in false negative or indeterminate results [3]. Consequently, NAb assays in general are quite susceptible to the presence of circulating drug as low as in the range of nanograms per milliliter.

Current efforts in overcoming drug interference are mainly focused on removing drug from a sample using approaches that require pretreatment of

samples under acidic conditions to disassociate drug–antibody complex, followed by separation of NAbs from drug with an affinity column utilizing the drug target or monoclonal neutralizing antibodies against the drug [4]. The NAb-containing column eluate presumably devoid of drug may be tested in the NAb assay for the detection of NAbs. While the success of this approach has been reported in some exemplary systems [5], there are several practical issues associated with this approach: (a) drug-removal steps significantly lower the assay's throughput; (b) large amounts of drug-depleting reagents such as the drug target or ADAs are needed but are not always easily obtainable; (c) assay optimization for the drug-removal process is required for different assays because a unified approach or protocol is not always applicable and therefore additional work is needed in the development and validation of a NAb assay; (d) during the drug-removal process, NAbs could potentially also be removed—for instance, for a monoclonal antibody drug, if only one arm of the antibody binds to a NAb and the other arm is unoccupied (which is likely to occur when drug is present in excess), removal of the drug could also remove the drug-bound NAb from the serum sample; and (e) the effects of sample treatment process (e.g., acid dissociation of the drug–antibody complex) on the neutralizing activity of the NAbs is not fully understood, as some NAbs may lose their neutralizing capability after such treatment. These issues make the drug-removal approach undesirable as a general approach for tackling drug interference issues for NAb assays.

21.3. PRINCIPLES OF DQ-NAB ASSAYS

Aside from the technical issues mentioned in Section 21.2 for the approaches used to remove drug, merely detecting whether NAbs are present in a sample may not provide relevant clinical information resulting from the formation of neutralizing antibodies. As mentioned earlier, the significance of NAb detection is related to potential clinical implications of the NAbs, namely, their impact on drug exposure and safety particularly if they can cross-react with an endogenous counterpart. Such a clinical implication is dependent on the level of circulating drug present at the time of testing. For instance, when drug is present at high levels, NAbs may inhibit only a small fraction of the total drug, resulting in little or no impact on drug exposure. On the other hand, when circulating drug levels are low, the presence of NAbs could significantly impact drug exposure; when NAbs are present in excess, they could potentially further inhibit the activity of endogenous counterpart and not only abolish the drug's activity but also inhibit the biological pathways mediated by its endogenous counterpart. Therefore, it is most meaningful to evaluate NAbs in the context of circulating drug levels, to provide clinically relevant information.

The recently described DQ-NAb approach provides (i) information about the level of biologically active drug present in a sample and (ii) the presence of "effective" NAbs (NAbs exhibiting neutralizing capability in the presence

of circulating drug) in a single assay format that is based on the quantitative analysis of bioactive drug concentrations [6]. It shares the same principle of the conventional NAb assay that inhibition of a biological readout associated with a known concentration of drug would indicate the presence of NAbs. In the DQ-NAb approach, a sample is first tested in a quantitative bioassay to measure the concentration of biologically active drug in the sample. Detection of biologically active drug levels indicates the absence of "effective" NAbs. If biologically active drug is not detectable, a selected concentration of drug is added to the sample, and recovery of this exogenously added drug is assessed. The presence of clinically "effective" NAbs is expected to result in an impairment of the quantitative recovery of the added drug concentration. An immunodepletion of the sample utilizing protein G/L beads could be performed to confirm that the impairment of the recovery of the added drug concentration is indeed due to ADAs (Fig. 21.2) .

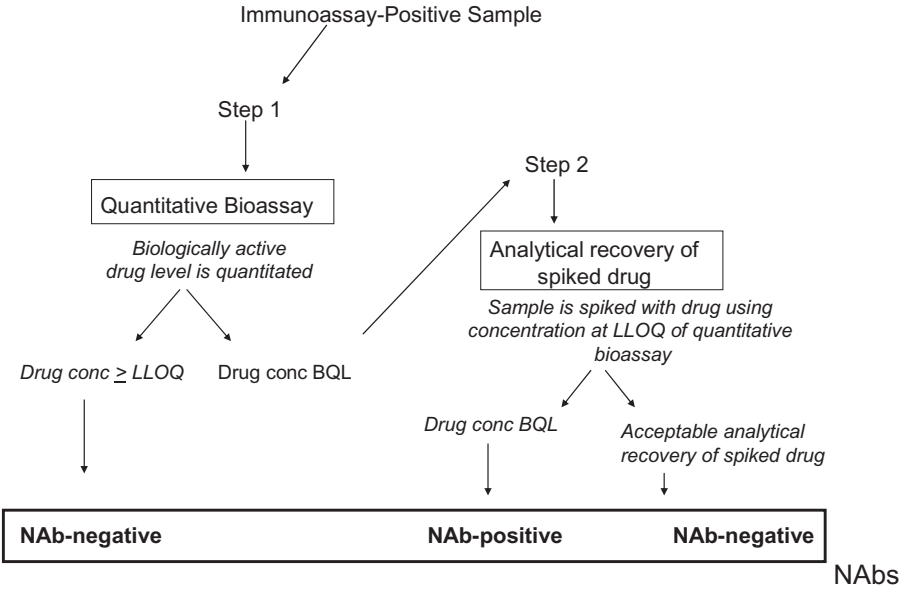

Fig. 21.2 Testing scheme for an immunoassay-positive sample using the DQ-NAb approach. One aliquot of the test sample is tested in the quantitative bioassay to determine the endogenous level of circulating drug in the sample. If detectable levels greater than the LLOQ of the assay are detected, the sample is assigned a negative result. If the drug concentration is below the quantifiable limit (BQL) of the assay, another aliquot of the sample is spiked with a concentration of the drug that represents the LLOQ of the quantitative bioassay and tested for analytical recovery of the added drug concentration. If the drug concentration is BQL in Step 2, the sample is assigned a positive result; this may be further confirmed by an immunodepletion approach using protein G/L, and so on. However, if an acceptable analytical recovery of the added drug concentration is observed, the sample is considered to be negative for NAbs.

21.4. FACTORS TO CONSIDER FOR CHOOSING A DQ-NAB ASSAY FORMAT

21.4.1. Testing Strategy

The decision to use a DQ-NAb assay instead of a conventional NAb assay requires careful assessment. If a conventional NAb assay is available that shows acceptable sensitivity and suitability for supporting a drug development program, it is recommended as the first choice for inclusion in the immunogenicity testing strategy. A non-cell-based NAb assay may also be used if a conventional cell-based NAb assay is not feasible. However in certain cases, where conventional and/or non-cell-based NAb assay sensitivity, matrix interference, et cetera, pose problems, the DQ-NAb approach may provide a suitable alternative for NAb detection. Adhering to the standard immunogenicity testing strategy, the DQ-NAb assay would be conducted on samples found to be positive in the ADA immunoassay. Since immunoassays benefit more than NAb assays from drug-dissociation and removal steps, the combination of these results with the drug bioactivity quantitation data from the DQ-NAb approach provides conclusive information if antibodies have impacted the biological activity of the drug in circulation.

21.4.2. Assay Characteristics

The DQ-NAb assay is only applicable to cell-based assays; therefore, the ability to use this approach is dependent upon the availability of a cell line that demonstrates a reliable response to the drug in the target species serum assay matrix. Reliability may be gauged by the dynamic range of the drug's dose–response curve and the accuracy and precision of the standard curve points.

21.5. ASSAY DEVELOPMENT AND VALIDATION

21.5.1. Step 1: Quantitative Bioassay

As mentioned earlier, the DQ-NAb assay uses a two-step approach, in which the first step measures biologically active levels of circulating drug in test samples. Assay development of the quantitative bioassay involves selection of an appropriate cell line that maintains its responsiveness to drug (if it is an agonist) or ligand (if the drug is an antagonist) in test species serum. Drug dose–response curves should be prepared in undiluted target species serum. The final assay matrix should constitute the serum concentration that is best tolerated by the cell line. Design of a drug's dose–response curve requires optimization to select the standard curve concentrations that, using a four-parameter logistic (4PL) model, yield acceptable accuracy and precision estimates. The upper and lower asymptotes, slope, and linear regions of the curve

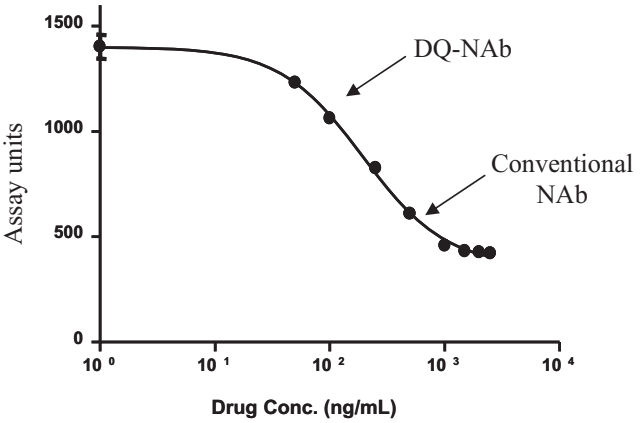

Fig. 21.3 A representative drug curve showing the concentrations that may be used to select the drug concentrations to develop a DQ-NAb or conventional NAb assay format.

should be determined. Independently prepared quality control (QC) samples spanning the linear portion of the curve should be identified and tested for the accuracy and precision of estimates using the 4PL fit. The lowest QC sample may be used to represent the assay's lower limit of quantitation (LLOQ). The latter concentration is a suitable one to use in the second step of the DQ-NAb approach when a sample does not show detectable levels of biologically active drug and NAbs may be suspected (Fig. 21.2). Assessing the analytical recovery of the drug concentration at the LLOQ of the quantitative assay maximizes assay sensitivity for NAb detection (Fig. 21.3).

Assay validation involves conducting drug standard curves on multiple days that include QC samples on the same plates (Fig. 21.4). Intra-run and inter-run precision and accuracy estimates are determined using appropriate statistical approaches. The assay's upper and lower limits of quantitation (ULOQ and LLOQ, respectively), representing the assay's range of reliable quantitation, require validation. The specificity of the assay system may be evaluated by comparing the effect of a drug-specific neutralizing antibody and/or an irrelevant antibody on the analytical recovery of one or more concentrations of drug spiked into test species serum.

21.5.2. Step 2: Analytical Recovery of the LLOQ Concentration in Test Samples

The second step of the DQ-NAb approach involves determination of the analytical recovery of the drug concentration corresponding to the LLOQ of the quantitative assay. This concentration may be spiked into 10–15 donor sera to assess the analytical recovery, and criteria may be set for acceptance. Hu et al. [6] evaluated the analytical recovery of the drug product at the LLOQ

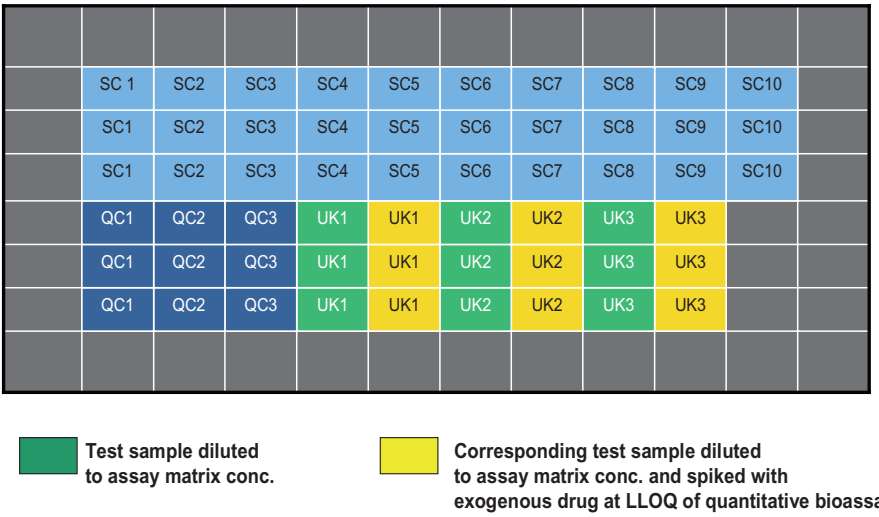
SC 1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
SC1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
SC1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
QC1 QC2 QC3 UK1 UK1 UK2 UK2 UK3 UK3
Test sample diluted to assay matrix conc.
Corresponding test sample diluted to assay matrix conc. and spiked with exogenous drug at LLOQ of quantitative bioassay

Fig. 21.4 A representative plate map that may be employed for conducting a DQ-NAb assay. SC1 to SC10 represent a multipoint drug standard curve prepared in undiluted target species serum. QC1–QC3 represent independently prepared quality control samples used to accept the performance of the drug standard curve. UK1–UK3 represent test samples for NAb analysis. The number of standard curve points and the replicates per point depend on the dynamic range and performance of the standard curve used in the assay. (See color insert.)

concentration in individual donor serum that either lacked or contained a positive control anti-drug neutralizing antibody. It was noted that the samples that contained antibody showed impaired recovery of the spiked drug concentration. The scope of this approach can be further expanded to evaluate recovery of the spiked drug concentration in samples that contain various ratios of drug–NAb mixtures. Hu et al. [6] noted that a NAb-to-drug ratio of 3:1 interfered with the analytical recovery of the spiked drug concentration in the quantitative bioassay

21.5.3. Assay Sensitivity of a DQ-NAb Assay

Assays used to detect ADAs utilize an assay cut point that is generally a statistically derived value [1, 2]. Depending on the assay format and the drug's mechanism of action, if NAbs are expected to result in a lowering of the assay signal, a positive result would be assigned to a sample if it yields a value lower than the derived assay cut point. Conversely, if the NAbs are expected to cause an increase in the assay signal, a positive result would be assigned to a sample if it yields a value higher than the assay cut point in the conventional NAb assays. For DQ-NAb assays, **assay sensitivity** is defined as the lowest concentration of the positive control antibody that shows an impairment of analytical

recovery of spiked drug concentration at the LLOQ of the quantitative bioassay. The sensitivity of the DQ-NAb assays may also be determined in terms of a NAb-to-drug ratio that interferes with the recovery of the spiked drug concentration; however, as is the case with all immunogenicity assays, these would only provide an estimate of the assay capability due to the use of an artificial positive control antibody (typically affinity purified serum from hyperimmunized animals).

21.6. PROS AND CONS OF DQ-NAB ASSAY

Although the DQ-NAb approach has better utility for the detection of neutralizing antibodies in the presence of high levels of drug, such as in the case of monoclonal antibody therapeutics, the same concept is potentially also applicable to other biotherapeutics. Although the DQ-NAb assay shares the same rationale as the conventional NAb assay in using a fixed concentration of drug for detecting NAbs, this approach may be able to provide better assay sensitivity especially for bioassays that show a narrow dynamic range in the drug's dose–response curve. Conventional NAb assays typically show better performance if there is a fivefold or greater change in assay signal when cells are treated with the selected drug concentration to obtain an assay response range to observe an inhibitory effect due to NAbs [2]. For the DQ-NAb assay the concentration that coincides with the LLOQ of the quantitative assay may be lower than the concentration that may be ideal for the conventional NAb assay format for the same drug product (Fig. 21.3). The use of a lower drug concentration enhances assay sensitivity, which is an issue for cell-based NAb assays in general. Also, the use of a quantitative approach for assessing the inhibition of the biological activity of the drug due to NAbs provides a better gauge of assay variability. In the DQ-NAb assay, sample results are back-calculated based on a drug standard curve on the same plate (Fig. 21.4); therefore interplate and interday variability can be normalized based on the standard curve performance. The normalization with a calibration curve provides an improvement over normalization with a single control data point as in the conventional NAb assay. Consequently, small changes in assay readout can be distinguished and higher sensitivity for NAb detection can be achieved with the DQ-NAb assay. Therefore, in addition to providing information on bioactive drug concentration, the DQ-NAb assay approach can support NAb detection with better sensitivity than the conventional NAb assay, while maintaining a single assay format.

The DQ-NAb assay does require a functional cell-based assay that is robust enough for quantitative analysis and demonstrates a good dynamic range. Cell-based assays are inherently variable and tend to be susceptible to matrix interference; in many cases, the ranges of biological readout are also rather limited. Therefore a good cell line with a robust drug-influenced biological readout is a prerequisite for the successful establishment of such an assay. The

development and optimization of assay conditions to establish a reliable drug dose–response curve is an important step in establishing a DQ-NAb assay format. Cell line engineering may be needed to pave the way for establishing robust assays when a suitable cell line is not readily available.

While the DQ-NAb assay provides information on bioactive drug concentration, it has not been evaluated as a suitable format for determining the quantitative level or titers of NAbs. A modification of the approach described by Grossberg et al. [7] may be employed to derive a DQ-NAb assay format that could provide additional quantitative data about the neutralizing capacity of the detected antibodies. Quantitative information about the levels of NAbs provides useful insight into the kinetics of NAb development and their relationship to clinical efficacy across the duration of a study or between studies.

21.7. CONCLUSIONS

The DQ NAb assay approach represents a novel strategy to secure clinically relevant information associated with the development of neutralizing antibodies when detection in the conventional NAb assay might be hindered due to the presence of high level of circulating drug in a sample. This approach is applicable to all types of biotherapeutics, including monoclonal antibodies, and may have utility in both preclinical and clinical studies.

REFERENCES

1. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V, Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
2. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
3. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
4. Patton, A., Mullenix, M.C., Swanson, S.J., Koren, E. (2005). An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J Immunol Methods*, *304*, 189–195.
5. Lofgren, J.A., Wala, I., Koren, E., Swanson, S.J., Jing, S. (2006). Detection of neutralizing anti-therapeutic antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J Immunol Methods*, *308*, 101–108.

6. Hu, J., Gupta, S., Swanson, S.J., Zhuang, Y. (2009). A bioactive drug quantitation based approach for the detection of anti-drug neutralizing antibodies in human serum. *J Immunol Methods*, *345*, 70–79.
7. Grossberg, S.E., Kawade, Y., Grossberg, L.D. (2009). The neutralization of interferons by antibody. III. The constant antibody bioassay, a highly sensitive quantitative detector of low antibody levels. *J Interferon Res*, *29*, 93–104.

factors. The potential impact of these factors on the immunogenicity of therapeutic proteins is well described in the literature. Hence, for every biopharmaceutical an individual risk category is defined and the preclinical and clinical immunogenicity program is designed according to individual needs in terms of assay strategy and timing. [1–4]

Orally administered microbial digestive enzymes share the product-related risk factors with other therapeutic proteins of microbial origin. However, the impact of those product attributes differs in view of the specific host-related factors in PEI. Since the oral route of administration and local action in the gastrointestinal tract are key elements for immunogenicity risk evaluation, we will first describe the relevant aspects of the intestinal absorption of macromolecules as well as the physiological and pathological mucosal immune responses. Based on these factors, we conclude that microbial digestive enzymes for the treatment of PEI have a low risk of causing adverse immune reactions. Should such immune reactions occur, however, hypersensitivity represents the most likely clinical consequence. Although this article focuses on the immunogenicity assessment of microbial digestive enzymes for oral replacement therapy in PEI, the general principles may be applicable to other orally ingested and locally acting microbial biopharmaceuticals.

20.2. PANCREATIC EXOCRINE INSUFFICIENCY

The pancreas has two main functions: the production of hormones such as insulin (endocrine activity) and the production of digestive enzymes (exocrine activity). In patients with PEI the exocrine function is affected and the synthesis or release of pancreatic enzymes into the pancreatic juice is reduced, resulting in maldigestion and malabsorption of nutrients. Associated clinical symptoms include weight loss, diarrhea, abdominal pain and loose, frequent stools containing unabsorbed fat (steatorrhea), and failure to thrive in children. Severe PEI occurs in certain diseases affecting the pancreas, including cystic fibrosis, Shwachman–Diamond syndrome, pancreatitis, status after pancreas surgeries, and cancer of the pancreas. Cystic fibrosis and Shwachman–Diamond syndrome are the most common causes of exocrine pancreatic insufficiency in children and chronic pancreatitis and post-pancreatectomy in adults [5]. We will focus on patients with cystic fibrosis and chronic pancreatitis as they are the best diagnosed and investigated populations in terms of maldigestion due to PEI.

Cystic fibrosis (CF) is a rare lethal inherited disease in Caucasians, occurring in about 1in 2000–2500 births [6]. The pancreatic dysfunction resulting from the blockage of the pancreatic ducts is already present at birth and CF patients span all age groups from neonates to adults. It is a life-threatening and chronically debilitating disease markedly impairing quality of life. CF is an autosomal recessive disorder caused by mutations in a single gene of chromosome 7, which encodes the cystic fibrosis transmembrane conductance

regulator (CFTR) [7]. CFTR is a chloride ion channel on the apical membrane of epithelial cells. Loss-of-function mutations in this gene result in abnormalities of cAMP-regulated ion transport across epithelia. The altered exchange of chloride and water across the epithelial layer results in viscous secretions, most importantly in the lung, intestine, and pancreas. The sticky mucus blocks the pancreatic duct thereby preventing the release of the pancreatic enzymes into the small intestine. Approximately 85–90% of all CF patients have clinical symptoms of maldigestion and require oral enzyme substitution from the day of birth [8]. Clinical symptomatology depends on genotype and phenotype [9].

Chronic pulmonary infections are common among cystic fibrosis patients. The dysregulated mucus production and transport obstruct the airways, favoring colonization by bacteria or fungi. The sustained infections and the vigorous local inflammatory responses are the major causes of morbidity and mortality in patients with CF [10].

Chronic pancreatitis (CP) is commonly defined as a chronic inflammatory process of the pancreas, characterized by irreversible morphological changes. In these patients PEI arises secondarily to the loss of acinar cells or obstruction of the pancreatic ducts and ductiles [11]. The progress of CP is a gradual one; it often takes 5 to 10 years for symptoms of maldigestion to occur [12]. Many cases in the Western world are attributed to alcohol abuse, but other causes include pancreatic duct obstruction from strictures or tumors, genetic mutations, and metabolic disorders such as hypertriglyceridemia and hypercalcemia [13–14]. CP is the most frequent cause of PEI in adults and does not alter the immune status of the patient.

20.3. ORAL ENZYME REPLACEMENT THERAPY

Enzymes used in oral replacement products such as prolyl endoprotease for treatment of celiac disease [15], β-D-galactosidase for treatment of lactose intolerance [16], and digestive enzymes for treatment of PEI [17] are supposed to act in the lumen of the gastrointestinal tract. In addition to replacement therapy, microbial enzymes are ingested during everyday life, as they are commonly used in the food industry or as dietary supplements [18].

In PEI, exogenous enzymes from natural sources or recombinant enzymes are used to replace absent or insufficient amounts of pancreatic enzymes in the upper intestine. The pancreatic juice contains a complex mixture of digestive enzymes, including amylase, lipase, and inactive precursors of proteases (procarboxypeptidase, proelastase, trypsinogen, chymotrypsinogen). In the small intestine the proteolytic enzymes are released from their precursors through cleavage by trypsin, whereas trypsin itself is activated by enterokinase and autoactivation. Polysaccharides with α-1,4-glucan bonds such as starch are hydrolysed into disaccharides through the action of amylase. The lipase breaks down lipids (triacylglycerols) into diacylglycerols, monoacylglycerols, free fatty

acids, and glycerol, while the proteases, once activated, degrade proteins into oligopeptides and single amino acids.

Several products containing digestive enzymes have been authorized for the treatment of PEI. The enzymes are administered as solid oral dosage forms, with every meal or snack, throughout life. Some examples are porcine pancreatic enzyme extracts such as Creon (Abbott), Zenpep (Eurand), Pancrease (Johnson&Johnson), Panzytrat (Axcan), and Pangrol (Berlin Chemie). These pharmaceuticals currently represent standard therapy for the treatment of PEI. In addition, microbial products such as Combizym (Daiichi-Sankyo) and Nortase (Repha) are available for the treatment of digestive disorders.

The microbial digestive enzyme products for the therapy of PEI have to ensure adequate digestion of dietary fat, protein, and carbohydrates. Therefore the products contain in general a mixture of digestive enzymes such as lipase, amylase, and protease. A prerequisite for the restriction to a limited number of active components is a broad substrate specificity for the individual enzymes used to replace the complex composition of natural pancreatic extracts. Microbial enzymes are selected due to the salient features of certain fungal and bacterial enzymes when compared with human pancreatic enzymes. They hydrolyze a wide range of substrates, are active throughout a wide pH range, and in addition are highly stable [19, 20]. Further optimization of enzyme efficacy and safety comprises, for example, altering the amino acid sequence by deletion of mycotoxin genes, proteolytic cleavage sites, and potential epitopes. Increased stability in the gastric compartment and resistance to proteases can also be achieved by optimizing formulations, for example by cross-linking microbial enzymes [21]. Enzymatic stability is required to ensure sufficient resistance to proteolytic cleavage by remaining endogenous proteases as well as to proteases within the administered enzyme mixture. By virtue of higher acid stability compared with established porcine pancreatic enzyme products, microbial enzymes might not require a functional coating for protection during passage through the stomach. The broader pH activity profile enables microbial enzymes to be active not only in the duodenum but also in the upper stomach directly after meal intake. Most important, this feature allows functionality in PEI patients affected by an acidified duodenum due to impaired pancreatic bicarbonate secretion.

The intrinsic properties of a protein preparation can markedly impact its potential immunogenicity. Such properties include size, protein sequence, post-translational modifications, formulation, impurities, and stability. The molecular weight of microbial enzymes ranges from about 20 kDa to 60 kDa (monomers). In general, microbial enzymes share little sequence homology with human lipases, amylases, or proteases. Hence, anti-drug antibodies are unlikely to cross-react with endogenous enzymes. With regard to post-translational modification, the degree of glycosylation and the presence of glycan epitopes vary as a function of the individual enzyme selected and both may impact the immunogenic potential of the protein. Specific anti-drug immunoglobulin E (IgE) antibodies directed against glycan epitopes have the

potential to cross-react with other glycated proteins. In addition the innate immune response against fungi can be triggered by β-glucans themselves [22]. For example, wild-type fungal enzymes are often highly glycosylated. Observed product-related substances and impurities such as aggregates, protein modifications and host-cell contaminants may also have a potential impact on the immunogenicity of the product. However, the potential degradation of these impurities during gastric passage in combination with trace systemic absorption diminishes the risk of adverse immune reactions.

Microbial digestive enzymes are produced in fungal as well as bacterial expression systems. The currently selected production organisms are generally not associated with an elevated risk of the development of allergies or other adverse immune reactions [18, 23]. Most of them have been used for centuries in the food industry and are known to be nontoxigenic and nonpathogenic. Common expression systems include *Aspergillus oryzae*, *Aspergillus niger*, *Rhizopus oryzae*, *Bacillus licheniformis*, and *Bacillus subtilis*. Some of the published strains and microbial digestive enzymes are reported in an official "Generally Regarded As Safe" (GRAS) affirmation as direct human food ingredient or used in food processing (available at http://www.cfsan.fda.gov/~rdb/opa-gras.html). It is also noteworthy that the Complementary Medicines Evaluation Committee of the Australian Competent Authority, the Therapeutic Goods Administration, has endorsed the suitability of microbial digestive enzymes as ingredients in listed medicines (see http://www.tga.gov.au/docs/pdf/cmec/cmecmi47.pdf). A comprehensive overview of microbial enzyme preparations used in food processing was provided by Pariza and Johnson [18]. Host-cell proteins (HCP) are present in the final product in a very limited and controlled amount. After ingestion they are degraded during stomach passage and gastrointestinal (GI) transit. If absorption of intact HCP does occur, it is likely that systemic plasma levels are negligible.

In summary, microbial digestive enzymes often display a higher degree of resistance to a low pH environment and proteolytic cleavage compared with the majority of ingested proteins. The foreign sequence and potential xenogenic post-translational modifications increase the immunogenicity potential of microbial enzymes but reduce the risk for autoreactivity of any potential anti-drug immune response. Furthermore, as we will discuss in the following sections, the impact of these product-related risk factors on the patient's safety is diminished by the oral route of administration.

20.4. ABSORPTION OF MACROMOLECULES IN THE GASTROINTESTINAL TRACT

The mucosal surface of the gastrointestinal tract has a selective permeability and acts as a protective barrier against harmful microorganisms and toxic proteins [24]. In addition to the absorption of hydrolyzed food constituents, small amounts of macromolecules are also taken up [25]. The process serves

to sample luminal antigens for recognition by the immune system and may elicit beneficial mucosal immune responses that are discussed later (see Section 20.5). Absorption of intact macromolecules or immunogenic fragments has been studied with poorly digestible proteins such as ovalbumin or β-lactoglobulin, especially when applied as large bolus. The absorption rate for stable proteins was estimated, based on the above reference proteins, to be in the range of one-hundredth of one percent or less of the ingested dose [24, 26]. Comparable trace absorption levels can be anticipated for the orally administered microbial digestive enzymes.

The microbial enzymes aid digestion in the stomach and small intestine. During further passage through the intestinal tract they are likely to be inactivated and degraded, similar to the fate of endogenous enzymes in healthy individuals [27, 28]. In the case of enzyme mixtures containing protease, based on the nature of the product and the GI transit time, the enzymes can additionally be cleaved over time by the microbial protease itself. The resulting small peptides or single amino acids, which are nonimmunogenic, are absorbed in the small intestine together with the hydrolyzed food constituents. Compared with the majority of the dietary proteins, which are rapidly degraded in the stomach and the duodenum, the microbial enzymes will remain intact for an extended period of time.

It is the fraction of intact proteins or large fragments entering the circulation that determines the extent of interactions with the systemic immune system and the probability of provoking adverse humoral- or cell-mediated systemic immune responses. Hence, it is generally recommended to investigate the systemic absorption and clearance of the intact therapeutic protein during development using appropriate assays. The magnitude of absorbed intact proteins depends upon the permeability of the gut, which is influenced by factors such as age, nutritional status, mucosal immunity, and gastric or intestinal function [25]. Under pathologic conditions the absorption rate may be increased and may lead to a predisposition for the development of allergies or intestinal inflammation. Altered absorption has been reported in neonates and upon oral antibiotic treatment, upon neutralization of gastric pH, during intestinal inflammation, or in patients with increased intestinal permeability.

Several studies have shown increased intestinal permeability in CF patients of all age groups [29–31] and in adults with PEI caused by diseases other than CF [32]. The cause for the increased permeability remains unclear, however, but may be a consequence of gastric acid hypersecretion, alteration in the intestinal microflora, or leaky junctions in the endothelial cell layer. Furthermore, the intestinal transit time is prolonged in CF patients [33, 34]. It is of particular relevance for the overall immunogenicity risk assessment that a predisposition for food allergies is not associated with CF [35]. However, a study by Smyth et al. [36] demonstrated elevated activation of the immune system in the gastrointestinal mucosa in CF children compared with controls. CF patients also have a higher prevalence of intestinal infections and inflammations [36–38]. In this context, the function of digestive enzyme supplements

as potential anti-inflammatory drugs has been discussed. Digestive enzyme supplements reduce the antigenic load in the upper intestine through efficient hydrolysis of potential immunogenic food components [39, 40]. The immunogenic potential of the microbial digestive enzymes themselves is discussed in the following section.

20.5. PHYSIOLOGICAL MUCOSAL IMMUNE RESPONSES

Upon ingestion the microbial enzymes are confronted with the mucosal immune system. The striking difference from parenteral biopharmaceuticals facing the systemic immune system is that the mucosal immune system is tuned to tolerate harmless antigens from food constituents or the commensal intestinal flora. The oral administration of antigen in the absence of immune-alerting "danger signals" generally induces a state of antigen-specific hyporesponsiveness of the immune system, known as "oral tolerance" [41–43]. In contrast, vigorous immune responses are mounted to pathogenic microbes that penetrate the mucosal barrier. The uncontrolled immune reactivity to harmless antigens can induce immunopathology that ranges from inflammatory bowel disease to allergies [44, 45].

The prime site for the generation of immune responses toward food and microbial antigens are the Peyer's patches in the small intestine and the solitary lymphoid follicles in the small and large intestine [41, 43]. Both are organized structures of immune cells directly underlying the epithelial surface. Microfold (M) cells, which are dispersed in the endothelial lining of the Peyer's patches, are specialized to transcytose large particles, for example, whole proteins and microbial constituents [46]. Those cells presumably are the source of the serum levels of ingested proteins, in addition to vesicular transport through epithelial cells or passive transport following damage to the endothelial barrier.

Specialized antigen-presenting cells, for example. tolerogenic dendritic cells that are in close proximity to the M cells and epithelial cells, take up the transcytosed material [43]. After processing, the antigens are presented to local T cells or to T cells that reside in the draining mesenteric lymph nodes. The presentation of harmless antigens derived from the commensal intestinal flora or food constituents generally results in anergy of the reactive T cells or induces the generation of T cells with regulatory properties. Such regulatory T cells in turn have the capacity to actively suppress the residual antigen-specific immune cells [43, 47]. At high oral doses of antigen, reactive T cells may also be deleted via apoptotic mechanisms [41].

Next to the control of T-cell reactivity, the induction of protective mucosal IgA prevents inflammatory responses to harmless luminal antigens. B cells recognizing the antigen become activated and eventually differentiate into antibody-secreting plasma cells guided by instructive signals in the microenvironment [48]. In a physiological mucosal immune response the B cells are predominantly induced to secrete IgA and to a lesser extent IgG. The instructed

B cells leave the site of origin, for example, the Peyer`s patches or secondary lymphoid organs, and disperse throughout the gastrointestinal mucosa guided by locally expressed homing-receptors [49, 50]. During their migration the cells terminally differentiate into plasma cells. Those long-lived B cells constantly produce immunoglobulins independent of the further presence of antigen. The produced IgA is efficiently secreted into the lumen as dimers or oligomers [51]. IgA constitutes only a minor part of serum immunoglobulins, but it is the predominant isotype in a number of external secretions, such as saliva and the intestinal fluid, followed by IgM [51]. Approximately 80% of all plasma cells in humans are located in the intestinal tissue and accordingly the production of IgA (40–60 mg/kg/day) exceeds the sum of production of the other immunoglobulin isotypes combined [50]. IgA plays a central role in the mucosal defense and in immune exclusion of harmless antigens.

The recognition of microorganisms and dietary proteins by the dimeric or oligomeric IgA does not elicit an inflammatory response due to the inability of this isotype to fix and activate the complement cascade. The binding of secreted IgA in the lumen prevents the antigen from adhesion to and penetration of the mucosal barrier [50–52]. Luminal constituents that cross the mucosa are captured by the IgAs produced in the mucosa and transported back via the IgA secretory pathway. The lowered absorption in turn reduces the activation of new antigen-specific T or B lymphocytes. The discovery of IgA-binding receptors expressed on the luminal cell surface of M cells points to a complementary mechanism [53]. The receptors may allow for uptake of small quantities of luminal IgA-bound antigen and channeling to antigen-presenting cells. The immune exclusion of the majority of antigen in combination with a controlled presentation of recognized luminal antigen may allow for a homeostatic production of IgA and sustained regulation of T-cell responses.

One part of the theoretical immunogenicity risk assessment for orally administered therapeutic proteins is the evaluation of IgA induction. Here it is an important fact that IgAs display abundant cross-reactivity [54, 55]. The commensal microorganisms and presumably also food constituents induce nonspecific IgA (natural IgA) and IgA specific to the corresponding antigen (specific IgA) [51, 52]. Therefore it can be concluded that the induction of an IgA response to the ingested microbial enzymes or the pre-existence of cross-reactive IgAs may occur. As previously elaborated, those IgAs binding to the product in the lumen would not alert the immune system and impact the safety of the patient. Moreover, those immunoglobulins would further reduce the absorption of the enzymes. The aforementioned IgA-mediated feedback loops are likely to result in a steady-state mucosal immune response. Since large quantities of the enzymes are active in the duodenum it can be anticipated that the impact of mucosal anti-drug immune responses on efficacy is negligible. In conclusion, development of ADA assays for the detection of specific mucosal IgA in feces samples are not clinically relevant and thus not recommended for routine analysis.

Next to the mucosal IgA, the induction of systemic immunoglobulins is also part of physiological immune responses to luminal constituents. In healthy individuals, dietary antigen-targeting IgAs and IgGs are commonly observed in the serum. Consequently, seroconversion of a fraction of patients receiving recombinant microbial digestive enzymes is expected. In contrast to specific serum IgG or IgA, which only at high levels may be indicative of aberrant immune reactivity, detectable serum IgE levels to a particular dietary antigen can be associated with allergies to the respective food [56–59].

20.6. ALLERGY AND IMMUNOGLOBULIN E

Immunoglobulin E–mediated hypersensitivity constitutes the major part of the adverse immunological reaction toward ingested harmless proteins that are generally referred to as food allergies [60]. Thus, it is recommended that the assessment of allergenicity should be a principal component of the immunogenicity strategy for the microbial digestive enzyme preparations

There is good experimental evidence that a deficiency or bypass of oral tolerance mechanisms results in hypersensitivity [60, 61]. IgE-dependent mechanisms are implicated in the majority of food allergic reactions, although cell-mediated immune reactions may be involved in certain conditions, for example, celiac disease. The development of IgE-mediated allergic reactions starts with the initial exposure to antigen or a structurally related protein triggering the generation of B cells that produce immunoglobulins of the IgE isotype. Upon re-exposure, the recognition of the allergen by the cognate IgE bound on the surface of mast cells and basophils leads to cross-linking of the antibodies and initiation of an intracellular signaling cascade that eventually results in the release of proinflammatory histamine and leukotrienes. Clinical symptoms often occur within minutes following the ingestion of the allergen and range from mild intestinal irritation, respiratory-related symptoms, or urticaria to life-threatening anaphylactic shock [60].

The prevalence of food allergies is higher in young children (approximately 5%) compared with an estimated 3–4% in adults in westernized countries [60]. Particularly in neonates, a marked increase in immune reactivity toward dietary proteins is observed that may be attributed to immaturity of the intestinal immune system or increased permeability of the endothelial barrier. Often the allergy is outgrown during childhood, with the exception of some allergens (i.e., peanuts, nuts, and seafood). Also in adults avoidance of the allergens may result in tolerance and the loss of allergic symptoms upon re-encounter of the antigen [62]. The therapeutic approach to (re)establish tolerance through oral administration of incremental amounts of the food allergen has shown some promising results, but so far no general protocol for oral desensitization exists [63].

The vast majority of reported allergic responses to food are attributed to a restricted number of food components. The major allergens in these foods

have been identified and have similar characteristics. They are abundantly present in the respective food and have an increased resistance to breakdown by the digestive enzymes and acidic conditions, resulting in a substantial part reaching the small intestine in an immunologically intact state [64]. This also holds true for microbial digestive enzymes. Further information about potential immunogenicity of the product is gained by sequence homology searches with known allergens and by evaluation of the organism used for production of the product, which might be a source of allergens. Cross-reactivity has to be considered when there is 35% identity over a sequence of 80 amino acids or for an identical sequence of 8 contiguous amino acids [65, 66]. This bioinformatic search is limited to linear epitopes, and identified sequence homologies need to be further evaluated for clinical relevance. Hence, this approach is most relevant for minimizing risk during early development.

Previous oral exposure to homologous enzymes or antigens derived from the host could impact the allergenic potential of the microbial enzyme product in the target population. Microbial enzyme preparations are widely used in the food industry. Therefore, tolerogenic pre-exposure to small amounts of potentially cross-reactive enzymes or HCP in food may occur. But in most cases the amounts will be insufficient to induce oral tolerance. Ingestion of microbial digestive enzymes contained in food, so far, has not been associated with induction of allergic responses. The conclusion of a well-controlled investigation of the allergenicity of 19 different commercial enzymes, including microbial lipase, protease, and amylase derived from expression systems of fungal origin, such as *Aspergillus oryzae* and *Aspergillus niger*, or of bacterial origin, such as *Bacillus licheniformis* and *Bacillus subtilis*, was that the "ingestion of food enzymes in general is not considered to be a concern with regards to food allergy" [23].

In contrast, prior sensitization via the airways to homologous microbial enzymes or the organism used for production may provoke allergic responses when taking the microbial enzymes. Despite a GRAS classification for many microbial lipases, amylases, and proteases used in laundry products, the food industry, and dietary supplements, it is known that they can cause occupational allergies via the respiratory route [67–69]. Another example is the α-amylase from *Aspergillus oryzae*, which is used to improve bread quality. The enzyme can cause asthma in bakers and workers in industry. A couple of food allergy cases after ingestion of bread containing α-amylase have also been reported [70, 71]. Allergic reactions in sensitized patients due to occupational exposure to industrial microbial enzymes will only affect single individuals who should, depending on the severity, refrain from using the respective microbial digestive enzyme product.

Besides the enzymes, remaining host-cell proteins in the product could elicit allergic responses especially in individuals with pre-existing IgE against the production organism or related species. Allergic bronchopulmonary aspergillosis (ABPA) due to occupational exposure to an *Aspergillus oryzae*–derived starter used in the fermentation of a soybean product is a reported example of sensitization by inhalation [72]. If a filamentous fungi is used as the expres-

sion system for the microbial digestive enzymes, potential cross-reactivity to pathogenic species, in particular to *Aspergillus fumigatus*, also needs to be taken into consideration. The primary causal agents for ABPA are antigens derived from a different *Aspergillus* species, namely, *Aspergillus fumigatus*. ABPA is associated with asthma or cystic fibrosis. In patients with cystic fibrosis the incidence of ABPA is approximately 10% [73], and filamentous fungi can be recovered from the lungs of around 30% of the patient population [74]. The colonization of the airways, most notably with *Aspergillus fumigatus*, causes excessive immune responses that include induction of IgE, which can be detected by skin-prick tests [75, 76].

However, for microbial digestive enzyme products, as for all other oral applied proteins, results of diagnostic methods have to be interpreted carefully. The development of IgE antibodies is not always predictive for the appearance of allergic symptoms upon oral antigen challenge [23, 77].

20.7. PROBABILITY AND CONSEQUENCES OF ANTI-DRUG ANTIBODIES TO MICROBIAL DIGESTIVE ENZYMES

The occurrence of anti-drug antibodies and the severity of clinical sequelae is specific to the intrinsic properties of the therapeutic protein and affected by a number of patient- and disease-related factors. The evaluation of probability and consequences of an immune response is the basis for immunogenicity risk assessment and defines the respective risk level. As a result this individual immunogenicity risk level determines the timing and extent of the anti-drug antibody (ADA) testing program. [1–4]

20.7.1. Probability of Anti-Drug Antibody Induction

The product-related factors per se imply that microbial digestive enzymes are generally immunogenic. However, despite the intrinsic immunogenicity of the product, the host-related factors, which will be discussed later, diminish the risk of adverse immune reactions. Critical factors for the immunogenicity evaluation of microbial digestive enzymes include the route of administration, local action in the GI tract, underlying disease, altered physiology of the gastrointestinal tract, age, and previous exposure to related products. The main product- and host-related factors and their influence on the probability of ADA induction are summarized in Table 20.1.

Most important, the mechanism of action of recombinant enzymes for treatment of PEI is to facilitate digestion in the stomach and small intestine. Although microbial pancreatic replacement products contain fungal or bacterial protein that is expected to be immunogenic in humans when administered parenterally, absorption into the systemic circulation is rather limited—or even negligible—following oral administration. Moreover, oral tolerance mechanisms are anticipated to effectively down-modulate host-related immune responses to orally administered therapeutic proteins.

TABLE 20.1 Probability of ADA Induction by Microbial Digestive Enzymes Based on Product- and Host-Related Factors

	Probability of ADA Induction
Product-Related Factors	
Protein class: enzymes	High
Origin: fungal or bacterial	High
Post-translational modifications: xenogenic, glycosylation	
Low sequence homology to human enzymes	
Stability in GI tract	High
Host-Related Factors	
Oral route of administration	Low
Chronic dosing	Low
No detectable or trace systemic exposure	Low
Patient subgroup: poor nutritional status	Low
Patient subgroup: neonates and small children	Increased
Patient subgroup: sensitized	High
Patients with increased intestinal permeability	Increased
Concomitant medication	Without impact

Cystic fibrosis and chronic pancreatitis patients share a number of host-related risk factors with a low probability of inducing anti-drug antibodies. First, the concomitant treatment is unlikely to have a significant overall impact on the probability of developing an adverse immune reaction to ingested microbial enzymes. With respect to lung function, treatment of CF aims at normalizing mucus secretion, controlling pulmonary infections, and preventing excessive immune responses [10, 78]. Immunomodulatory medication includes antibiotics and anti-inflammatory drugs. To normalize digestive functions and improve nutritional status, fat-soluble vitamin supplements and digestive enzyme preparations are administered, often together with acid-blocking agents. Medication such as acid blockers may have an impact since acid-induced degradation of immunogenic food constituents is reduced, but this may be counteracted by the anti-inflammatory medication for treatment of lung disease. Second, the administration schedule, that is, the chronic administration with every meal, lowers the risk of developing ADAs. Furthermore the poor nutritional status observed in some patients prior to onset of therapy is likely to diminish their immune reactivity.

The increased intestinal permeability is more prominent in patients with CF than those with CP. This condition is likely to elevate the probability of ADA responses. Considering the correlation of age with the immune reactivity to ingested proteins, neonates and young children with CF have a further increased probability for seroconversion. However, the impact of increased intestinal permeability seems to be low since the prevalence for food allergies is not increased in CF [35].

20.7.2. Consequences of Anti-Drug Antibodies to Microbial Digestive Enzymes

As elaborated in Section 20.7.1, it can be anticipated that ADAs may appear in a significant portion of CF and CP patients during chronic treatment. This would not necessarily imply a negative impact as a potential consequence in the case of systemic binding antibodies or specific non-neutralizing mucosal IgA. As an example, binding anti-drug antibodies without correlation to clinical symptoms were detected in CF patients on long-term treatment with porcine pancreatic extract. Specific anti-porcine trypsin IgGs were detected in sera of 63% of CF patients after chronic dosing, but no correlation to clinical symptoms was apparent [79]. Moreover, the long history of safe use of animal-sourced pancreatic enzyme products suggests that oral tolerance will be the most probable response to oral administration of digestive enzymes. Reported hypersensitivity reactions are rare and generally mild and transient.

In contrast to parenteral biopharmaceuticals, especially those with human sequences and endogenous counterparts, there are some striking differences that will not be discussed here in detail. Immediate infusion reactions do not apply to oral products. Nonacute consequences, such as immune complex–mediated reactions, are not expected for most microbial digestive enzymes due to trace systemic bioavailability and lack of endogenous counterparts. Due to their physiological action, the digestion of food, severe adverse reactions such as overstimulation (formation of superagonists) are not relevant.

In view of the phenomenon of food allergy, hypersensitivity reactions to microbial digestive enzymes may occur in some individuals. Hypersensitivity reactions to orally administered proteins are most frequently IgE-mediated, for example, type I reactions summarized as the "oral allergy syndrome." Other types of allergic reactions such as immune-complex or cell-mediated complications are less commonly observed. Hence, potential adverse reactions after administration of microbial digestive enzymes are skin-related symptoms (e.g., rash, urticaria, rhinitis, angioedema), respiratory-related (e.g., asthma) symptoms, or intestinal disorders (e.g., abdominal pain, vomiting, diarrhea). The time course in general spans from immediate reactions (ranging from itching in the mouth to gastrointestinal disturbance and asthma) to late-phase reactions in the range of 24–48 hours after ingestion that are cell mediated. Late-phase reactions are usually linked to acute-phase reactions, such as gastrointestinal symptoms. Similar observations have been made with porcine pancreatic enzyme extracts, for which rare hypersensitivity cases include rhinitis, conjunctivitis, urticaria, angioedema, asthma, or intestinal disorders [77]. These symptoms are manageable and, if observed after treatment with microbial enzymes, alternative treatment would be available. Anaphylaxis, a potentially severe or fatal reaction to re-exposure of a drug is a rare event in humans [80]. Furthermore, databases such as PubMed and Pharmapendium do not report any cases of anaphylaxis after ingestion of microbial digestive enzyme products.

TABLE 20.2 Consequences of Anti-Drug-Antibodies and Relevance for Microbial Digestive Enzymes

Potential Consequence	Probability
Infusion reaction	Not applicable
Interference with PK assays (enzymatic activity in chyme)	Not applicable
Interference with PD markers	Not applicable
Overstimulation of endogenous mechanisms (superagonist)	Not applicable
Neutralization of endogenous counterpart (deficiency syndrome)	Low
Loss of efficacy	Low
Serum sickness or immune complex disease (IgG, IgM)	Low
Autoimmune reaction	Low
Hypersensitivity	Medium
Interference with absorption assays (ELISA, plasma samples)	High

PK, pharmacokinetic; PD, pharmacodynamic.

Besides clinical consequences, ADAs in general can influence absorption studies and related immunoassays. Due to the local action in the intestine, secreted ADAs of the IgA isotype could principally influence efficacy of microbial digestive enzymes. However, the impact is likely to be negligible relative to the amounts of enzymes administered. As for other therapeutic proteins, ADA interference with bioanalytical assays has to be considered during assay development. The consequences of ADAs to the microbial digestive enzymes and their relevance are summarized in Table 20.2.

In summary, patients suffering from PEI have a low probability of developing adverse immune reactions to ingested microbial enzymes. As discussed for occupational exposure, for some individuals, such as atopic patients or patients with pre-existing specific IgE, induction of adverse immune reactions should be considered. In the context of ABPA, the potential cross-reactivity of the production organism to pathogenic microorganisms has to be considered. Patients suffering from ABPA are pre-exposed and already sensitized to filamentous fungi. Those patients have to be monitored closely to evaluate the clinical relevance of any observed cross-reactivity.

Based on the overall probability and consequences of potential adverse immune reactions, microbial digestive enzymes for treatment of PEI can be classified as low-risk biopharmaceuticals for most patients. As a consequence, it is recommended to monitor systemic binding ADAs during development but with a reduced immunogenicity program.

20.8. ASSAY STRATEGY

Given the low risk of adverse immune responses, the assay strategy may be limited to a preclinical screening assay and clinical evaluation of systemic ADAs with a focus on IgE-mediated allergic responses. The implementation

of neutralizing antibody assays for mucosal IgA is not recommended; rather the evaluation of pharmacodynamic parameters should be an integral part of the immunogenicity assay strategy.

20.8.1. Preclinical Studies

Preclinical immunogenicity studies for human or humanized biopharmaceuticals are considered to have low predictive value but are crucial for adequate study interpretation and for head-to-head comparison of immunogenicity in comparability studies [80–84]. For microbial digestive enzymes that are "non-self proteins" for both the toxicology species and humans, preclinical immunogenicity testing may give some indication about the frequency and type of immune reaction to be expected.

Systemic ADAs are an important endpoint in toxicity studies for interpretation of (i) pharmacokinetic data, (ii) pharmacodynamic data, (iii) adverse reactions [81], and (iv) potential systemic exposure in concentrations below the limit of the bioanalytical assays. Screening assays should be suitable for the detection of systemic binding antibodies, but isotype characterization is not mandatory. In addition to ADA assessment, clinical symptoms, hematology, and histology of the lymphoid organs will complete the preclinical immunogenicity evaluation. The Göttingen minipig is a suitable nonrodent species, with the advantages of having a comparable physiology of digestion and being a good model of human immunology [85, 86]. During assay development and validation it is advantageous to use minipig serum samples prepared in the actual toxicology laboratory to evaluate the appropriateness of the validation parameters (i.e., cut point, sensitivity, minimal dilution). Especially for the minipig species it is useful to include hemolytic samples, which might need special treatment prior to analysis.

20.8.2. Absorption Assays

Even if systemic exposure is expected to be low, it is recommended to evaluate systemic absorption as part of the development program. In this context, enzyme-linked immunosorbent assays (ELISAs) are usually preferable due to their higher sensitivity and selectivity compared with enzyme activity assays. These immunoassays have to be developed for every individual enzyme present in the final product and have to be validated for every matrix used in preclinical and clinical studies, as described by DeSilva et al. [87] and Findlay et al. [88]. Since generally only very low amounts of intact macromolecules are absorbed (plasma levels in the range of 0.01% of the oral dose), assay sensitivity has to be high. During assay development it has to be demonstrated that the antibodies used for detection are specific for the individual enzyme and do not cross-react with the other enzymes in the preparation. In addition determination of potential assay interference due to the presence of anti-drug antibodies is in general part of the development phase.

20.8.3. Pharmacokinetics, Pharmacodynamics, and Neutralization Assays

The *in vivo* pharmacokinetic and pharmacodynamic studies are generally conducted in preclinical models such as pancreatic duct–ligated pigs [89] or human intubation studies [90] by analysis of intestinal luminal samples. These studies are in general enzymatic assays and not discussed here in detail. In addition reliable pharmacodynamic markers, for example, the coefficient of fat absorption for lipase, are available.

Antibody-mediated changes in systemic exposure due to antibodies that enhance systemic clearance or antibodies that slow clearance could occur. Antibodies that increase the rate of drug clearance are not clinically relevant due to the local site of action in the GI tract. Antibodies that slow clearance as described for cytokines and hormones [91, 92] can potentially prolong systemic exposure. Due to the trace systemic absorption, it can be anticipated that antibodies that slow clearance are not clinically relevant for microbial enzyme replacement products. Potential interference with the absorption assay has to be considered for all samples containing anti-drug antibodies.

For microbial digestive enzymes, which are administered together with food, neutralizing immune responses would only be relevant at the site of action, that is, in the stomach and small intestine. But even if IgA with some neutralizing capacity were generated here, it is highly unlikely that the local immunoglobulin concentration would be sufficient to neutralize a significant fraction of enzymes in view of the high concentration of enzymes present. Reduced efficacy upon repeated dosing would be recognized clinically by a noticeable increase in PEI symptomatology, such as diarrhea, flatulence, bloating, abdominal pain, and steatorrhea. For these patients, an alternative treatment is available with pancreatic enzymes extracted from pig pancreas (pancreatin), which is currently standard therapy. Thus, the development of neutralization assays is not relevant unless there is a correlation between a confirmed positive ADA response and a substantially reduced efficacy; monitored by changes in the pharmacodynamic marker, for example, the coefficient of fat absorption for microbial lipase.

20.8.4. Anti-Drug Antibody Assays

As a result of the its xenogenic origin, a fraction of patients may develop a systemic antibody response toward the microbial digestive enzyme product. Preclinical and clinical monitoring of ADA responses is necessary to establish a potential link between the systemic immunoglobulins and potential adverse drug reactions and to investigate whether any systemic exposure to the drug product has actually occurred. It is recommended to investigate the incidence and titer of the antibody response predose and post-treatment and at adequate intervals after repeated dosing. Accordingly, appropriate methods have to be developed and validated to enable the detection of host ADAs. Detailed description is provided in the literature [87, 93–95] and in regulatory guidance

[80, 83, 84]. These methods can be adopted for assessing the systemic immune response to microbial digestive enzymes in preclinical and clinical studies.

As for bioanalytical assays, ADA assays have to be developed and validated for every individual enzyme present in the medicinal product, and for every matrix used in preclinical and clinical studies. For immunogenicity assays and for bioanalytical assays, it is equally important that a representative batch of the individual enzyme is used for generating the relevant polyclonal detection antibodies, containing the relevant variants and drug-related impurities. Clearly this preparation can only be a surrogate of the constituents absorbed *in vivo* since it mainly consists of the full-length enzyme in the drug product. Due to technical limitations it cannot represent the complete set of fragments generated during digestion in the gastrointestinal tract. Various assay formats are suitable (e.g., Biacore or ELISA assays), but special attention has to be drawn to the fact that enzyme substrate and inhibitors might be present in the matrix and could induce conformational changes. Another point that requires attention is the proteolytic or autolytic activity of the protease in the product. Both can result in severe matrix effects and assay interference. In contrast to parenteral biopharmaceuticals, drug interference in ADA assays is in general not an issue for orally applied macromolecules due to their trace systemic absorption. In a pharmacokinetic study in pigs, plasma (pro)colipase and trypsin(ogen) levels remained unchanged after oral administration of porcine pancreatic enzyme extract, and a rapid elimination of pancreatic lipase activity was detected after intravenous application [96].

In terms of immunoglobulin isotype characterization, ADA assays for clinical samples should be able to detect all immunoglobulin isotypes but, based on the treatment schedule and risk assessment, detection of specific IgM is judged to be only of minor relevance. In contrast, it is recommended to establish analytical methods for the characterization of specific IgE (see Section 20.8.5) with the aim of supporting the correlation of the systemic immune responses with potential clinical symptoms of hypersensitivity. For the design of preclinical studies it has to be considered that analysis of systemic IgA is not predictive for potential development of neutralizing mucosal IgA in humans. There are reported species differences for the source of systemic and mucosal IgA [51, 97]. Since mucosal IgA is not clinically relevant and the development of (pre)clinical ADA assays for the detection of specific mucosal IgA in feces or serum samples is complex, they are in general not included in the assay strategy as a routine approach.

20.8.5. Allergenicity Assessment

Currently, there is no validated preclinical model that will predict the allergic potential of recombinant microbial enzymes upon oral administration. Animal models under investigation for evaluation of food allergens include mice, rats, guinea pigs, atopic dogs, and neonatal swine. These models are used for *ranking* different food allergens, for example, based on IgE (total and specific) responses

and clinical symptoms after re-exposure. A selection of animal models for assessing allergenicity of novel proteins is reviewed by Helm [86].

In clinical studies IgE can be detected by serological *in vitro* tests such as radioallergosorbent test (RAST), Immuno-CAP, and basophil histamine release tests or by *in vivo* skin prick test (SPT) (see Chapter 12). In some cases it could be demonstrated that the magnitude of a specific IgE correlates with the probability of generating an allergic response [98]. Methods for these tests are found in the literature [99, 100]. For study design the following parameters have to be considered: availability of detection antibodies, study length, number of subjects, type and frequency of measurements, and target population (atopics, ABPA); patients can either be predisposed or never have been exposed to the enzymes. A positive result in a diagnostic test proves that there was sufficient exposure of the microbial digestive enzyme, or cross-reactive antigens, to stimulate a detectable IgE-mediated response in the tested individual. However, as for orally administered proteins in general, it has to be noted that positive samples in these (IgE) assays must be interpreted carefully. These assays test high local concentrations of the microbial enzymes and residual HCPs. Hence, they are not always predictive for the likelihood of developing allergic symptoms after oral antigen ingestion, in view of trace absorption and potential rapid degradation in the gastrointestinal tract. In addition RAST or SPT may be positive and misleading due to clinically insignificant serological cross-reactions. It is well known that some individuals with, for example, positive SPT have specific IgE but never develop allergic symptoms. Poor correlation of positive SPT with allergic symptoms in oral challenge tests was observed for bacterial and fungal enzymes [23]. Other examples are positive SPTs following animal-derived pancreatic enzymes in CF patients that could not be correlated with GI symptoms [77]. In addition, for enzyme mixtures the irritation potential of the product itself has to be considered as capable of causing false positive results, especially for products containing proteases.

20.9. CONCLUSION

The immunogenicity risk for microbial digestive enzyme products is diminished by the oral route of administration and their local action in the stomach and small intestine. Severe consequences such as neutralization, immune complex–mediated reactions, cross-reactivity with an endogenous counterpart, or generation of superagonists are not relevant for most microbial digestive enzymes due to their local action in the GI tract, trace systemic bioavailability, low sequence homology to endogenous counterparts, and pharmacological mechanisms. Based on the probability of inducing ADAs together with the low potential of severe consequences, microbial digestive enzymes for the treatment of PEI can be generally classified as low-risk biopharmaceuticals. Experiences with animal-sourced pancreatic enzyme products underline the

assumption that oral tolerance will be the most probable immune response. There is a long history of safe use for those products, and hypersensitivity reactions are rare and generally mild and transient.

Despite a low level of systemic absorption following oral administration, it can be anticipated that a fraction of patients will develop an ADA response over time, given the intrinsic immunogenicity of a microbial enzyme product. The theoretical risk for ADA induction is dependent on the patient's age and disease, with elevated risk for young children with CF. This would not necessarily imply a negative impact, as shown for systemic antibodies specific to food antigens and for systemic ADAs without clinical relevance in CF patients treated with porcine pancreatic extracts. Hence, it is recommended that appropriate clinical risk-management measures be applied until the requisite data have been obtained. The probability of a clinically significant immune response in the target population following chronic administration remains to be substantiated during subsequent stages of the clinical development program for every individual microbial digestive enzyme product. The required testing program will vary with the type and origin of the enzymes, the chosen formulation, and the target population.

For therapeutic proteins, existing regulatory guidance and scientific literature focus on immunogenicity risks and consequent assay strategies upon systemic application. The risk-based approach for immunogenicity testing can be adopted for oral microbial digestive enzyme products. Here, interactions with the scientific community and concerned authorities during development are encouraged, to facilitate the design of an adequate immunogenicity testing program for the individual product. As a result of the classification as a low-risk biopharmaceutical, the immunogenicity assay program may be reduced to a screening assay for the relevant toxicology species, a clinical screening and confirmation assay for every enzyme in the preparation, and the characterization of IgE-mediated responses. In general it is recommended that the evaluation of allergenicity form an integral part of the immunogenicity risk assessment. Atopic subjects as well as subjects sensitized to related proteins, such as HCP (e.g. *Aspergillus*) or microbial enzymes (e.g., occupational exposure), should also be identified and closely monitored since hypersensitivity reactions have to be considered.

REFERENCES

1. Koren, E., Smith, H., Shores, W., Shankar, G., Finco-Kent, D., Rup, B., Barett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S., Taniguchi, G., Zuckerman, L.A., Stebbins, C., and Mire-Sluis, A.R. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
2. Shankar, G., Pendley, C., Stein, K.E. (2007). A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. *Nature Biotechnol*, *25*, 555–561.

3. Shankar, G., Shores, E., Wagner, C., Mire-Sluis, A. (2006). Scientific and regulatory considerations on the immunogenicity of biologics. *Trends Biotechnol*, *24*, 274–280.
4. Schneider, C.K., Papaluca, M., Kurki, P. (2009). A European perspective on immunogenicity evaluation. *Nature Biotechnol*, *27*, 507–508.
5. Dror, Y., Freedman, M.H. (2002). Shwachman–Diamond syndrome. *Br J Haematol*, *118*, 701–713.
6. Ratjen, F., Döring, G. (2003). Cystic fibrosis. *Lancet*, *361*, 681–689.
7. Sinaasappel, M., Stern, M., Littlewood, J., Wolfe, S., Steinkamp, G., Heijerman, H.G.M., Robberecht, E., Döring, G. (2002). Nutrition in patients with cystic fibrosis: a European consensus. *J Cystic Fibrosis*, *1*, 51–75.
8. Durie, P.R., Forstner, G.G. (1989). Pathophysiology of the exocrine pancreas in cystic fibrosis. *J Roy Soc Med*, *82* (Suppl. 16), 2–10.
9. Durie, P.R. (1992). Pathophysiology of the pancreas in cystic fibrosis. *Netherlands J Med*, *41*, 97–100.
10. Frerichs, C., Smyth, A. (2009). Treatment strategies for cystic fibrosis: what's in the pipeline? *Expert Opin Pharmacother*, *10*, 1191–1202.
11. DiMagno, M.J., DiMagno, E.P. (2009). Chronic pancreatitis. *Curr Opin Gastroenterol*, *25*, 454–459.
12. DiMagno, E.P., Pap, A. (1994). Treating pancreatic insufficiency. *Contemp Int Med*, *6*, 72–86.
13. Khalid, A., Whitcomb, D.C. (2002). Conservative treatment of chronic pancreatitis. *European J Gastroenterol Hepatol*, *14*, 943–949.
14. Giger, U., Stanga, Z., DeLegge, M.H. (2004). Management of chronic pancreatitis. *Nutr Clin Pract*, *19*, 37–49.
15. Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., Koning, F. (2006). Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *Am J Physiol. Gastrointest Liver Physiol*, *291*, 621–629.
16. Montalto, M., Curigliano, V., Santoro, L., Vastola, M., Cammarota, G., Manna, R., Gasbarrini, A., Gasbarrini, G. (2006). Management and treatment of lactose malabsorption. *World J Gastroenterol*, *12*, 187–191.
17. Löhr, J.M., Hummel, F.M., Pirilis, K.T., Steinkamp, G., Körner, A., Henniges, F. (2009). Properties of different pancreatin preparations used in pancreatic exocrine insufficiency. *European J Gastroenterol Hepatol*, *21*, 1024–1031.
18. Pariza, M.W., Johnson, E.A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul Toxicol Pharmacol*, *33*, 173–186.
19. Griffin, S.M., Alderson, D., Farndon, J.R. (1989). Acid resistant lipase as replacement therapy in chronic pancreatic exocrine insufficiency: a study in dogs. *Gut*, *30*, 1012–1015.
20. Suzuki, A., Mizumoto, A., Sarr, M.G., DiMagno, E.P. (1997). Bacterial lipase and high-fat diets in canine exocrine pancreatic insufficiency: a new therapy of steatorrhea? *Gastroenterology*, *112*, 2048–2055.
21. Borowitz, D., Goss, C.H., Stevens, C., Hayes, D., Newman, L., O'Rourke, A., Konstan, M.W., Wagener, J., Moss, R., Hendeles, L., Orenstein, D., Ahrens, R.,

Oermann, C.M., Aitken, M.L., Mahl, T.C., Young, K.R. Jr, Dunitz, J., Murray, F.T. (2006). Safety and preliminary clinical activity of a novel pancreatic enzyme preparation in pancreatic insufficient cystic fibrosis patients. *Pancreas*, *32*, 258–263.

22. Sorrell, T.C., Chen, S.C. (2009). Fungal-derived immune modulating molecules. *Adv Exp Med Biol*, *666*, 108–120.
23. Bindslev-Jensen, C., Skov, P.S., Roggen, E.L., Hvass, P., Brinch, D.S., (2006). Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food Chem Toxicol*, *44*, 1090–1915.
24. Turner, J.R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Rev Immunol*, *9*, 799–809.
25. Udall, J.N., Walker, W.A. (1982). The physiologic and pathologic basis for the transport of macromolecules across the intestinal tract. *J Pediatr Gastroenterol Nutr*, *1*, 295–301.
26. Tsume, Y., Taki, Y., Sakane, T., Nadai, T., Sezaki, H., Watabe, K., Kohno, T., Yamashita, S. (1996). Quantitative evaluation of the gastrointestinal absorption of protein into the blood and lymph circulation. *Biol Pharmcol Bull*, *19* (Suppl.10), 1332–1337.
27. Layer, P., Holtmann, G. (1994). Pancreatic enzymes in chronic pancreatitis. *Int J Pancreatol*, *15*, 1–11.
28. Layer, P., Gröger, G. (1993). Fate of pancreatic enzymes in the human intestinal lumen in health and pancreatic insufficiency. *Digestion*, *54* (Suppl. 2), 10–14.
29. Gregory, P.C. (1996). Gastrointestinal pH, motility/transit and permeability in cystic fibrosis. *J Pediatr Gastroenterol Nutr*, *23*, 513–523.
30. vanElburg, R.M., Uil, J.J., van Aalderen, W.M., Mulder, C.J., Heymans, H.S. (1996). Intestinal permeability in exocrine pancreatic insufficiency due to cystic fibrosis or chronic pancreatitis. *Pediatr Res*, *39* (Suppl. 6), 985–991.
31. Leclercq-Foucart, J., Forget, P., Sodoyez-Goffaux, F., Zappitelli, A. (1986). Intestinal permeability to [^{51}Cr]EDTA in children with cystic fibrosis. *J Pediatr Gastroenterol Nutr*, *5*, 384–387.
32. Pezzilli, R., Barassi, A., Morselli-Labate, A., Fantini, L., Tomasetti, P., Campana, D., Casadei, R., Finazzi, S., Melzi D'Eril, G., Corinaldesi, R. (2007). Fecal calprotectin and elastase 1 determinations in patients with pancreatic diseases: a possible link between pancreatic insufficiency and intestinal inflammation. *J Gastroenterol*, *42*, 754–760.
33. Dalzell, A., Freestone, N., Billington, D., Heaf, D. (1990). Small intestinal permeability and oroceacal transit time in cystic fibrosis. *Arch Dis Childhood*, *65*, 585–588.
34. Bali, A., Stableforth, D., Asquith, P. (1983). Prolonged small intestinal transit time in cystic fibrosis. *Br Med J*, *287*, 1011–1013.
35. Ferguson, A., Merrett, T.G., Littlewood, J.M., Bolderson, I. (1986). IgE antibodies to foods are not a feature of cystic fibrosis. *Hum Nutr Clin Nutr*, *40*, 255–258.
36. Smyth, R.L., Croft, N.M., O'Hea, U., Marshall, T.G., Ferguson, A. (2000). Intestinal inflammation in cystic fibrosis. *Arch Dis Childhood*, *82*, 394–399.
37. Bruzzese, E., Raia, V., Gaudiello, G., Polito, G., Buccigrossi, V., Formicola, V., Guarino, A. (2004). Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. *Aliment Pharmacol Ther*, *20*, 813–819.

38. Raja, V., Maiuri, L., De Ritis, G. (2000). Evidence of chronic inflammation in morphologically normal small intestine of cystic fibrosis patients. *Pediatr Res*, *47*, 344–350.

39. Raithel, M., Weidenhiller, M., Schwab, D., Winterkamp, S., Hahn, E.G. (2002). Pancreatic enzymes: a new group of antiallergic drugs? *Inflamm Res*, *51* (Suppl. 1), 13–14.

40. Seidman, E.G., Singer, S. (2003). Therapeutic modalities for cow's milk allergy. *Ann Allergy Asthma Immunol*, *90*, (6) (Suppl. 3), 104–111.

41. Weiner, H.L. (1997). Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Immunol Today*, *18*, 335–343.

42. Chehade, M., Mayer, L. (2005). Oral tolerance and its relation to food hypersensitivities. *J Allergy Clin Immunol*, *115*, 3–12.

43. Tsuji, N.M., Kosaka, A. (2008). Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends Immunol*, *29*, 532–40.

44. Garrett, W.S., Gordon, J.I., Glimcher, L.H. (2010). Homeostasis and inflammation in the intestine. *Cell 19*, *140*, 859–870.

45. Strobel, S., Mowat, A.M. (2006). Oral tolerance and allergic responses to food proteins. *Curr Opin Allergy Clin Immunol*, *6*, 207–213.

46. Mowat, A.M. (2003). Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Rev Immunol*, *3*, 331–341.

47. Izcue, A., Coombes, J.L., Powrie, F. (2009). Regulatory lymphocytes and intestinal inflammation. *Annu Rev Immunol*, *27*, 313–338.

48. Cerutti, A. (2008). The regulation of IgG class switching. *Nature Rev Immunol*, *8*, 421–434.

49. Macpherson, A.J., McCoy, K.D., Johansen, F.E., Brandtzaeg, P. (2008). The immune geography of IgA induction and function. *Mucosal Immunol*, *1*, 11–22.

50. Fagarasan, S., Kawamoto, S., Kanagawa, O., Suzuki, K. (2010). Adaptive immune regulation in the gut: T cell–dependent and T cell–independent IgA synthesis. *Annu Rev Immunol*, *28*, 243–273.

51. Woof, J.M., Mestecky, J. (2005). Mucosal immunoglobulins. *Immunol Rev*, *206*, 64–82.

52. Rakoff-Nahoum, S., Medzhitov, R. (2006). Role of the innate immune system and host-commensal mutualism. *Curr Topics Microbiol Immunol*, *308*, 1–18.

53. Mantis, N.J., Cheung, M.C., Chintalacharuvu, K.R., Rey, J., Corthésy, B., Neutra, M.R. (2002). Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. *J Immunol*, *169*, 1844–1851.

54. Shimoda, M. (1999). Local antibody response in Peyer's patches to the orally administered dietary protein antigen. *Biosci, Biotechnol Biochem*, *63*, 2123–9.

55. Quan, C.P., Berneman, A., Pires, R., Avrameas, S., Bouvet, J.P. (1997). Natural polyreactive secretory immunoglobulin A autoantibodies as a possible barrier to infection in humans. *Infect Immun*, *65*, 3997–4004.

56. Hidvegi, E., Cserhati, E., Kereki, E., Savilahti, E., Arato, A. (2002). Serum immunoglobulin E, IgA, and IgG antibodies to different cow's milk proteins in children with cow's milk allergy: association with prognosis and clinical manifestations. *Pediatr Allergy Immunol*, *13*, 255–61.

57. De Jong, E.C., Van Zijverden, M., Spanhaak, S., Koppelman, S.J., Pellegrom, H., Penninks, A.H. (1998). Identification and partial characterization of multiple major allergens in peanut proteins. *Clin Exp Allergy*, *28*, 743–751.
58. Fallstrom, S.P., Ahlstedt, S., Carlsson, B., Lönnerdal, B., Hanson, L.A. (1986). Serum antibodies against native, processed and digested cow's milk proteins in children with cow's milk protein intolerance. *Clin Allergy*, *16*, 417–423.
59. Johansson, S.G., Dannaeus, A., Lilja, G. (1984). The relevance of anti-food antibodies for the diagnosis of food allergy. *Ann Allergy*, *53*, 665–672.
60. Sicherer, S.H., Sampson, H.A. (2010). Food allergy. *J Allergy Clin Immunol*, *125* (2) (Suppl. 2), 116–125.
61. Burks, A.W., Laubach, S., Jones, S.M. (2008). Oral tolerance, food allergy, and immunotherapy: implications for future treatment; *J Allergy Clin Immunol*, *121*, 1344–1350.
62. Sampson, H.A. (2003). Food allergy. *J Allergy Clin Immunol*, *111* (Suppl. 2), 540–547.
63. Skripak, J.M., Sampson, H.A. (2008). Towards a cure for food allergy. *Curr Opin Immunol*, *20*, 690–696.
64. Kimber, I., Dearman, R. (2002). Factors affecting the development of food allergy. *Proc Nutr Soc*, *61*, 435–439.
65. Gendel, S.M. (1998). Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. *Adv Food Nutr Res*, *42*, 63–92.
66. Hileman, R.E., Silvanovich, A., Goodman, R.E., Rice, E.A., Holleschak, G., Astwood, J.D., Hefle, S.L. (2002). Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int Arch Allergy Immunol*, *128*, 280–291.
67. Bernstein, J.A., Bernstein, D.I., Stauder, T., Lummus, Z., Bernstein, I.L. (1999). A cross-sectional survey of sensitization to *Aspergillus oryzae*–derived lactase in pharmaceutical workers. *J Allergy Clin Immunol*, *103*, 1153–1157.
68. Baur, X. (2005). Enzymes as occupational and environmental respiratory sensitisers. *Int Arch Occupational Environ Health*, *78*, 279–86.
69. Kempf, W., Oman, H., Wüthrich, B. (1999). Allergy to proteases in medical laboratory technicians: a new occupational disease? *J Allergy Clin Immunol*, *104*, 700–701.
70. Losada, E., Hinojosa, M., Quirce, S., Sánchez-Cano, M., Moneo, I. (1992). Occupational asthma caused by alpha-amylase inhalation: clinical and immunologic findings and bronchial response patterns. *J Allergy Clin Immunol*, *89*, 118–125.
71. Sander, I., Raulf-Heimsoth, M., Van Kampen, V., Baur, X. (2000). Is fungal α-amylase in bread an allergen? *Clin Exp Allergy*, *30*, 560–565.
72. Akiyama, K., Takizawa, H., Suzuki, M., Miyachi, S., Ichinohe, M., Yanagihara, Y. (1987). Allergic bronchopulmonary aspergillosis due to *Aspergillus oryzae*. *Chest*, *91*, 285–286.
73. Tillie-Leblond, I., Tonnel, A.B. (2005). Allergic bronchopulmonary aspergillosis. *Allergy*, *60*, 1004–1013.
74. Sudfeld, C.R., Dasenbrook, E.C., Merz, W.G., Carroll, K.C., Boyle, M.P. (2010). Prevalence and risk factors for recovery of filamentous fungi in individuals with cystic fibrosis. *J Cystic Fibrosis*, *9* (2), 110–116.

75. Laufer, P., Fink, J.N., Bruns, W.T., Unger, G.F., Kalbfleisch, J.H., Greenberger, P.A., Patterson, R. (1984). Allergic bronchopulmonary aspergillosis in cystic fibrosis. *J Allergy Clin Immunol*, *73*, 44–48.
76. O'Driscoll, BR., Powell, G., Chew, F., Niven, R.M., Miles, J.F., Vyas, A., Denning, D.W. (2009). Comparison of skin prick tests with specific serum immunoglobulin E in the diagnosis of fungal sensitization in patients with severe asthma. *Clin Exp Allergy*, *39*, 1677–1683.
77. Griese, M., Dokupil, K., Latzin, P. (2005). Skin prick test reactivity to supplemental enzymes in cystic fibrosis and pancreatic insufficiency. *J Pediatr Gastroenterol Nutr*, *40*, 194–198.
78. European Medicines Agency (EMEA). (2009). Guideline on the clinical development of medical products for the treatment of cystic fibrosis. Available at http://www.ema.europa.eu/pdfs/human/ewp/914708enfin.pdf
79. Quirk, P., Greer, R., Shepherd, R., Cleghorn, G. (1993). Serum immunoglobulin G directed against porcine trypsin in the serum of cystic fibrosis children receiving porcine pancreatic enzyme supplements. *J Paediatr Child Health*, *29*, 196–200.
80. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. (1997). Guideline preclinical safety evaluation of biotechnology-derived pharmaceuticals. Available at http://www.ich.org/LOB/media/MEDIA503.pdf
81. Ponce, R., Abad, L., Amaravadi, L., Gelzleichter, T., Gore, E., Green, J., Gupta, S., Herzyk, D., Hurst, C., Ivens, I.A., Kawabata, T., Maier, C., Mounho, B., Rup, B., Shankar, G., Smith, H., Thomas, P., Wierda, D. (2009). Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies. *Regul Toxicol Pharmacol*, *54*, 164–182.
82. Wierda, D., Smith, H.W., Zwickl, C.M. (2001). Immunogenicity of biopharmaceuticals in laboratory animals. *Toxicology*, *158* (1–2), 71–74.
83. EMA. (2007). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. Available at http://www.ema.europa.eu/pdfs/human/biosimilar/1432706enfin.pdf
84. Food and Drug Administration (FDA). (2009). Draft guidance for industry assay development for immunogenicity testing of therapeutic proteins. Available at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM192750.pdf
85. Rothkötter, H.J. (2009). Anatomical particularities of the porcine immune system—a physician's view. *Dev Comp Immunol*, *33*, 267–272.
86. Helm, R.M. (2002). Food allergy animal models. *Ann New York Acad Sci*, *964*, 139–150.
87. DeSilva, B., Smith, W., Weiner, R., Kelley, M., Smolec, J., Lee, B., Khan, M., Tacey, R., Hill, H., Celniker, A. (2003). Recommendations for the bioanalytical method validation of ligand-binding assays to support pharmacokinetic assessments of macromolecules. *Pharm Res*, *20*, 1885–1900.
88. Findlay, J.W., Smith, W.C., Lee, J.W., Nordblom, G.D., Das, I., DeSilva, B.S., Khan, M.N., Bowsher, R.R. (2000). Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. *J Pharm Biomed Anal*, *21*, 1249–1273.
89. Tabeling, R., Gregory, P., Kamphues, J. (1999). Studies on nutrient digestibilities (pre-caecal and total) in pancreatic duct–ligated pigs and the effects of enzyme substitution. *J Animal Physiol Animal Nutr*, *82*, 251–263.

90. Carrière, F., Grandval, P., Renou, C., Palomba, A., Priéri, F., Gaillo, J., Hennigess, F., Sander-Struckmeier, S., Laugier, R. (2005). Quantitative study of digestive enzyme secretion and gastrointestinal lipolysis in chronic pancreatitis. *Clin Gastroenterol Hepatol*, *3*, 28–38.
91. Aston, R., Cowden, W.B., Ada, G.L. (1989). Antibody-mediated enhancement of hormone activity. *Mol Immunol*, *26*, 435–446.
92. Rehlaender, B.N., Cho, M.J. (1998). Antibodies as carrier proteins. *Pharm Res*, *15*, 1652–1656.
93. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
94. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T., Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48* (5), 1267–1281.
95. Wadhwa, M., Thorpe, R. (2006). Strategies and assays for the assessment of unwanted immunogenicity. *J Immunotoxicol*, *3* (3), 115–121.
96. Gewert, K., Holowachuk, S.A., Rippe, C., Gregory, P.C., Erlanson-Albertsson, C., Olivecrona, G., Kruszewska, D., Piedra, J.V., Weström, B., Pierzynowski, S.G. (2004). The enzyme levels in blood are not affected by oral administration of a pancreatic enzyme preparation (Creon 10,000) in pancreas-insufficient pigs. *Pancreas*, *28*, 80–88.
97. Decaro, N., Pratelli, A., Tinelli, A., Martella, V., Camero, M., Buonavoglia, D., Tempesta, M., Caroli, A.M., Buonavoglia, C. (2004). Fecal immunoglobulin A antibodies in dogs infected or vaccinated with canine coronavirus. *Clin Diagnostic Laboratory Immunol*, *11*, 102–105.
98. Ahlstedt, S., Murray, C.S. (2006). In vitro diagnosis of allergy: how to interpret IgE antibody results in clinical practice. *Prim Care Respiration J*, *15*, 228–236.
99. Rusznak, C., Davies, R.J. (1998). ABC of allergies: diagnosing allergy. *Br Med J*, *316*, 686–689.
100. Nicholson, P.J., Newman Taylor, A.J., Oliver, P., Cathcart, M. (2001). Current best practice for the health surveillance of enzyme workers in the soap and detergent industry. *Occup Med*, *51*, 81–92.

of immunogenicity is achieved by a variety of techniques, for example, *in silico*, *in vitro*, *ex vivo*, and *in vivo*. *In silico* methods use bioinformatics to predict T- and B- cell epitopes [6]. In most cases, these predictions are then tested *in vitro* by use of T-cell or dendritic cell lines [7]. When blood from antigen-exposed patients is available, an *ex vivo* assay can be performed to test for reactivity with human T-cells [8]. Transgenic mouse models immune tolerant for the human therapeutic of interest are more and more used to assess immunogenicity and its underlying immunological mechanism [9–12]. One of the most common techniques to measure ADAs in transgenic animals is the enzyme-linked immunosorbent assay (ELISA). Since it was first described in the 1970s [13], it has become the most commonly used quantitative immunoassay in (immunogenicity) research using mice, rats, and rhesus monkeys [10, 12, 14–18].

ELISA is based on the activity of an enzyme that allows determining antigen or antibody concentration by the amount of converted substrate. It is a fast and reliable method and one that can easily and efficiently discriminate between responders and nonresponders (i.e., antibody-positive and antibody-negative individuals), toward the recombinant human therapeutic. Once the responders are identified, the immunological response can be analyzed by protein microarray analysis, EliSpot for cytokine secretion (especially interferon gamma, IFN-γ), affinity using Biacore, and neutralizing antibody assays [19, 20]. Due to its straightforward approach, ELISA is one of the first tools to measure anti-drug antibodies.

19.2.2. Experimental: ELISA

19.2.2.1. Bioassay Procedure. Anti-drug antibodies in mouse serum and plasma are measured with a bioassay based on the binding of ADAs to an absorbed antigen on an immunoplate, followed by incubation with a secondary antibody that carries the enzyme for substrate conversion [18]. The rate of color formation is proportional to the amount of ADA present in the serum or plasma. The following paragraph describes the experimental procedure of an ELISA used to detect ADAs in serum of mice treated with recombinant human therapeutic interferon beta [10, 18].

Test wells are coated with 100 µL of rh protein, diluted to a final concentration of 2 µg/mL in phosphate buffered saline (PBS). Incubation takes place overnight at 4°C, followed by washing with 0.05% Tween20 in PBS (PBS/T). To block unspecific binding sites, wells are filled with 300 µL of a blocking solution containing 4% milk and 0.1% Tween20 in PBS. Blocking takes place at room temperature (RT) for 2 hours, followed by washing with PBS/T. Serum or plasma samples are then added in a dilution of 1:100 in blocking buffer and incubated for another hour at RT. After another wash step, 100 µL of a secondary antibody containing the enzyme is added to each well and reaction is allowed to take place for an hour at RT. Horseradish peroxidase (HRP) is a frequently used enzyme, coupled to the detection antibody. Plates are then

subjected to intensive washing to remove any excess secondary antibody, which might lead to high background signals. The last wash is conducted with plain PBS to remove excess Tween20, which might interfere with the enzyme activity. Color conversion is initiated by adding 3,3′,5,5′-tetramethylbenzidine (TMB) and stopped by adding sulfuric acid. Color intensity is measured with an immunoplate reader at 450 nm wavelength. Each plate contains positive controls, which consist of serum or plasma from nontransgenic animals treated with the rh protein, and negative controls, which are collected from untreated nontransgenic animals. Samples are considered positive when the mean is at least threefold higher than the mean of the negative plus the typical 95th percentile of the standard deviation. After an initial screening, positive samples are titered out by a twofold dilution following the same ELISA protocol.

19.2.2.2. Evaluation of ADA Titers. The titer of the samples is obtained by plotting the absorbance values of the serial dilution against the log dilution. By using the program GraphPad Prism, Version 4.0, the values can be fitted to a nonlinear curve and the reciprocal of the dilution of the 50% endpoint (i.e., EC_{50} = concentration of antibody inducing half-maximal binding to antigen) value is considered the titer of the sample. A standard anti-rh protein serum is added to each plate.

19.2.3. Practical and Technical Issues

Immunogenicity mouse models have always been subjected to skepticism. However, the generation and validation of mouse models immune tolerant for human proteins creates a useful tool to look at the immunological mechanism leading to the formation of ADAs. For the first time, the immune reaction can be studied in a fully functional immune system environment rather then just testing the rh protein in an isolated cell line. Also, for *in vitro* studies, ELISA is a common tool to measure cytokine production. Within the animal models, ELISA is a quick and technically easy approach to measure and monitor the occurrence of ADAs. Though ELISA is a reliable technique, several technical issues occur during establishment of the ELISA method.

When using an ELISA to detect ADAs formed against a rh antibody, the availability of appropriate secondary antibodies that can discriminate between ADAs and rh antibody could be limited [21]. This is the case when a transgenic animal that produces human antibodies is given a rh antibody. Alternative detecting reagents such as radioactive-labeled or biotinylated therapeutic antibody would then be suitable [21].

Another issue regarding the ELISA is the induction of conformational changes when coating the rh protein to the immunoplate [22, 23]. Naturally occurring antibodies directed against such modified molecules can lead to high aspecific binding. A catching format could prove effective in such cases [22, 23]. Also, formulation contents of the rh therapeutic could hinder proper

binding to the ELISA plate; for example, the presence of human serum albumin in a protein formulation might compete with the drug for binding to the ELISA plate, thus lowering the sensitivity of the assay. Prior dialysis of the rh protein might be required to circumvent that problem.

Once the assay has been set up and validated, it can serve as a solid approach to measure formation of ADAs. In the case of the ELISA described in Reference 10, it should be mentioned that the results correspond to data acquired by neutralizing antibody assays [18, 19].

A opposed to human research, where confirmation assays are routinely used to validate ADA presence, confirmation assays are almost never included in animal research. A confirmation assay involves retesting of samples for ADA presence by, for example, radioimmunoassay (RIA) or radioimmunoprecipitation assay (RIPA). It reinforces the results gained by ELISA, and thus minimizes the number of false positives and negatives [24]. In patients, therapy is often adjusted when efficacy is low or when severe side effects are present. To link low efficacy and severe side effects to the presence of ADAs, it is vital to be "sure" of the ADA status of the patient [25–27], and thus confirmation assays are necessary. In the case of animal research, confirmation assays would allow the researcher to know the rate of false positives and negatives associated with his or her assay. When more certainty about ADA presence is needed, a confirmation assay should be used.

Mouse models immune tolerant for rh therapeutics represent a useful tool to asses and study immunogenicity in a fully functional immune system. In combination with ELISA and other assays, they will improve our understanding of immunogenicity and lead to safer and more efficient drugs.

19.3. DETECTING NEUTRALIZING ANTIBODIES

19.3.1. Real-Time RT-PCR Bioassay to Detect NAbs against Recombinant Human Interferon Beta in Immune Tolerant Transgenic Mice

In immunogenicity studies using a transgenic mouse model immune tolerant for recombinant human interferon beta (rhIFN-β), both wild-type and transgenic mice are immunized with different variants of rhIFN-β. Subsequently ADAs against rhIFN-β are detected by ELISA. Although ELISA assays are able to detect ADAs, they do not enable discrimination between merely binding antibodies (BAbs) and neutralizing antibodies (NAbs). For this reason, the development of novel neutralization assays for the measurement of NAbs, besides BAbs, in mouse serum represents an important step in transgenic mouse research as model for (preventing) NAb development in humans.

Measurement of anti-IFN-β BAbs in serum is fast and technically easy [28–32]. On the other hand, measurement of anti-IFN-β NAbs is technically difficult. Existing bioassays for NAb detection in human sera utilize either the antiviral activity of IFN-β (i.e., a cytopathic effect assay, CPE) [33] or the

specific induction of IFN-β-induced for myxovirus resistant protein A (MxA protein), that is, the MxA protein induction assay (MPA) [34, 35].

Recently, a novel neutralization assay has been developed that basically corresponds to the MPA, but is significantly better in terms of reliability due to the use of the highly reproducible and sensitive technology of real-time reverse transcriptase polymerase chain reaction (RT-PCR) [36]. The achievement of successful measurements of NABs in mouse serum has been possible just by using this latter protocol [19]. Indeed, although the CPE assay still represents the "gold standard" for NAb measurements in human sera [37], it has not been possible to use this bioassay for NAb detection in mice, due to the high cytotoxicity of mouse sera against A549 cells (i.e., a human cell line). Cytotoxicity is, in fact, critical for bioassays and, in particular, for cytopathic assays, where the critical endpoint is the cytopathic effect (i.e., number of dead cells) of viruses on cultured cells. It has been therefore necessary to focus on a different procedure for measuring anti-IFN-β NAbs in mouse serum. The final procedure is the real-time RT-PCR bioassay, which involves real-time RT-PCR technology to quantitate the inhibition of IFN-β-induced MxA mRNA expression: the presence of NAbs in serum reduces the amount of MxA transcript synthesized in response to a standard rhIFN-β dose. The extent of the specific gene induction, caused by dilutions of samples, allows a determination of the degree of neutralization and, thus, a NAb titration.

19.3.2. Experimental

19.3.2.1. Bioassay Procedure. Neutralizing antibody levels in mouse serum are assessed with a bioassay based on the inhibition of MxA gene expression in a human cell line (i.e., A549 cell line) [19]. Test wells are incubated with rhIFN-β diluted in culture media at 20 laboratory units per milliliter (LU/mL) and an equal amount of test serum in a serial twofold dilution (1:2.5 up to 1:5120), resulting in a final rhIFN-β concentration of 10 LU/mL. To determine the actual LU per milliliter in each test, simultaneous titrations to obtain rhIFN-β standard curves are run on every plate. In addition, since rhIFN-β1b binds approximately three times more NAb molecules than rhIFN-β1a, based on a MIU (million international units)-to-MIU comparison [28, 38], NABs are commonly tested against the type of rhIFN-β used for injecting the animal, in order to avoid false negatives.

After six and a half hours of incubation, samples are removed by aspiration and cells are lysed for RNA extraction and cDNA synthesis. Finally, target (MxA) and reference (eukaryotic 18S rRNA) genes are detected with a real-time RT-PCR multiplex assay. The response of targets is expressed in terms of relative expression considering the difference in threshold cycles (Ct) for target and reference (ΔCt).

Before proceeding with sample titration, negative samples are commonly screened out. In specific, screening samples are diluted 1:10, incubated with 20 LU/mL of rhIFN-β and tested for MxA gene expression. A sample with a

ΔCt value of less than 50% of the highest concentration on the standard curve is considered NAb negative. Otherwise, the sample is titrated.

19.3.2.2. Definition of NAb Units. The endpoint of the bioassay is the half-maximal MxA induction and is determined from a standard curve of rhIFN-β concentration and MxA induction; sigmoidal dose–response curves are generated from the MxA expression values determining the 50% endpoint (i.e., EC_{50} = concentration of rhIFN-β inducing half-maximal MxA induction).

The Kawade approach [39, 40], based on the mathematical model of antibody–antigen interaction developed by Kawade and recommended by the World Health Organization [33], is used for the computation and reporting of NAb results. In its current form it allows calculation of neutralizing antibody titer from the dilution of serum that reduces 10 LU/mL of rhIFN-β activity to 1 LU/mL. A refinement to the calculation has enabled the titer of antibody that reduces a fixed concentration of rhIFN-β (may be more or less than 10 LU/mL) to 1 LU/mL to be calculated from a Kawade formula as 10-fold reduction units per milliliter (TRU/mL) [41, 42].

19.3.3. Practical and Technical Issues

Through developing a transgenic hybrid mouse model immune tolerant for rhIFN-β, the barriers to the analysis of immunogenicity of a wide range of rhIFN-β products, and the structural factors influencing this immunogenicity, were overcome. By using the real-time RT-PCR bioassay, NAbs have been quantitated, for the first time, in serum from wild-type immunized mice, while, as expected, serum from nonimmunized mice was tested as negative. Notably, NAbs titers correlate with BAbs titers obtained by ELISA, indicating that the assay can be used as support of the ELISA measurement.

Clearly, the CPE assay still represents the "gold standard" for NAbs measurements in human sera, as recently stated by a European Federation of Neurological Societies (EFNS) scientific task force [37]. However, in the specific context of detecting NAbs in mouse serum, the real-time RT-PCR bioassay seems to be more reliable than the CPE assay for two main reasons: (1) any bias from cell density and cell viability can be easily checked and corrected thanks to normalizing genes and (2) lower incubation periods are needed (i.e., 6.5 hours instead of 24 hours).

Concerning the first point, the possibility of controlling for the amount of viable cells by normalizing to a reference gene represents an important advantage, particularly in light of the high cytotoxicity observed in mouse sera tested against human cells. Reference genes can also control for different input cell plating densities, differences in cell growth, or different expressivity in culture subjected to several passages. All these differences could undoubtedly affect the response, with greater total response by higher cell densities and vice versa, that is, more or less MxA/mL cell densities. Differences in MxA/mL cell densi-

ties can thus lead to significant variability between laboratories, as well as between tests performed in the same laboratory.

Changes in the reference gene expression were also studied in relation to cytotoxicity, a critical feature for any bioassays and, in particular, for cytopathic assays, where the critical endpoint is the cytopathic effect (i.e., number of dead cells) of viruses on cultured cells. As expected, shorter incubation times resulted in less cytotoxicity. A time-dependent protective effect was observed with the 6.5-hour incubation period because there was only a slight cytotoxicity (evidenced by higher Ct values of the reference gene) at high sera concentrations, that is, 1:10 and 1:20. Such slight cytotoxicity was, however, easily checked and corrected thanks to the normalizing process of the real time RT-PCR.

Variability is also decreased in that the real-time RT-PCR bioassay procedure involves the use of multiplex PCR. The amplification and detection of the cDNA target and reference in the same reaction tube offers many benefits such as reduced reagent costs (targets are amplified together instead of separately), reliable results (well-to-well variability is eliminated by co-amplification of the reference control), conservation of precious samples (more quantification data can be obtained per sample), and increased throughput (more targets can be analyzed per run on a real-time cycler).

All in all, the description of a novel neutralization assay for measuring NAbs against rhIFN-β in mice definitely represents a major step forward toward a way to examine the causes of immunogenicity of rhIFN-β in humans, by studying the whole immune response (i.e., both BAbs and NAbs) to rhIFN-β formulations in wild-type and transgenic immune tolerant mice. Without such a bioassay, it would hardly be possible to test any experimental procedure in mice for preventing NAb development in humans.

19.4. GENERAL CONSIDERATIONS

This chapter describes two types of antibody assays used in immunogenicity research in transgenic mice: the ELISA and a neutralizing assay. The ELISA is the major ADA detection and quantification assay used due to its straightforward approach, while the neutralizing assay presents a novel class of bioassays used in animal research that provides information on ADA function. However, several considerations do apply.

The first relates to ADA–drug complex interference. When assessing ADA levels or function in the presence of drug, ADA–drug complexes can lead to underestimation of ADA levels and neutralizing activity. In the case of ELISAs, improved assays have to be developed, which could include coating with more specific capture antigens, or serum/plasma needs to be pretreated to separate pre-existing ADA–drug complexes [43]. The interference of ADA–drug complexes can be minimized by adjusting injections and blood drawing schedule.

For example, pharmacokinetic studies in mice transgenic for human interferon beta show that the drug is cleared from the system before blood is drawn (data unpublished). However, absence of drug cannot be guaranteed in all studies, especially when using therapeutic rh antibodies. The half-life of such rh antibodies often limits detection of ADAs in the absence of drug. A possibility would be to test for ADAs after a washout period, or, develop antibody assays that can tackle the ADA–drug complex interference. In human research several promising attempts have been made to measure ADAs in the presence of drug; however, this topic still needs to be addressed in animal research.

An important consideration for the future is the standardization of antibody assays. Recommendations for strategies how to assess immunogenicity of rh therapeutics are present; however, no standardized ELISA techniques exist [4, 44]. Standardization initiatives in human research are in progress. The Fabry antibody standardization initiative has been started, which organizes the worldwide standardization of assays used to detect anti-galactosidase antibodies in patients suffering from Fabry disease [45]. Also, suggestions have been made to improve detection of ADAs against monoclonal antibody therapeutics [21]. Overall, standardization of antibody assays in animal (and human) research will allow better interpretation and comparison of immunogenicity data worldwide and thus lead to better, improved therapies.

19.5. CONCLUSION

Antibody assays in animal research enable researchers to study immunogenicity of therapeutic proteins during early stages of product development in an organism with an intact immune system. Additionally, these assays are vital in studying which immunological mechanisms underlie immunogenicity of biopharmaceuticals.

However, further optimization and standardization is needed to progress in both research areas, and thus lead to products with lower immunogenicity.

REFERENCES

1. European Medicines Agency (EMEA). (2007). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins, Ref. EMEA/CHMP/BMWP/14327/2006.
2. Wierda, D., Smith, H.W., Zwickl, C.M. (2001). Immunogenicity of biopharmaceuticals in laboratory animals. *Toxicology*, *158*, 71–74.
3. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R, Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
4. Shankar, G., Devanarayan, V., Amaravadi, L., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T.,

Quarmby, V., Smith, H., Smith, W., Zuckerman, L.A., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–1281.

5. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
6. Bryson, C.J., Jones, T.D., Baker, M.P. (2010). Prediction of immunogenicity of therapeutic proteins: validity of computational tools. *BioDrugs*, *24*, 1–8.
7. Mueller, R., et al. (2009). Evaluation of the immuno-stimulatory potential of stopper extractables and leachables by using dendritic cells as readout. *J Pharm Sci*, *98*, 3548–3561.
8. Aarskog, N.K., Maroy, T., Myhr, K.M., Vedeler, C.A. (2009). Antibodies against interferon-beta in multiple sclerosis. *J Neuroimmunol*, *212*, 148–150.
9. Fradkin, A.H., Carpenter, J.F., Randolph, T.W. (2009). Immunogenicity of aggregates of recombinant human growth hormone in mouse models. *J Pharm Sci*, *98*, 3247–3264.
10. Hermeling, S., Jiskoot, W., Crommelin, D., Bornaes, C., Schellekens, H. (2005). Development of a transgenic mouse model immune tolerant for human interferon beta. *Pharm Res*, *22*, 847–851.
11. Hermeling, S., Aranha, L., Damen, J., Slijper, M., Schellekens, H., Crommelin, D., Jiskoot, W. (2005). Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon alpha2b. *Pharm Res*, *22*, 1997–2006.
12. Hermeling, S., et al. (2006). Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. *J Pharm Sci*, *95*, 1084–1096.
13. Engvall, E., Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, *8*, 871–874.
14. Ottesen, J.L., et al. (1994). The potential immunogenicity of human insulin and insulin analogues evaluated in a transgenic mouse model. *Diabetologia*, *37*, 1178–1185.
15. Strayer, D.S., Kapp, J. (1982). Insulin-specific antibodies and insulitis in mice immunized with purified porcine insulin. *Clin, Exp, Immunol*, *47*, 77–84.
16. Zwickl, C.M., et al. (1991). Comparison of the immunogenicity of recombinant and pituitary human growth hormone in rhesus monkeys. *Fundam Appl Toxicol*, *16*, 275–287.
17. Zwickl, C.M., Hughes, B.L., Piroozi, K.S., Smith, H.W., Wierda, D. (1996). Immunogenicity of tissue plasminogen activators in rhesus monkeys: antibody formation and effects on blood level and enzymatic activity. *Fundam Appl Toxicol*, *30*, 243–254.
18. van Beers, M.M., et al. (2010). Hybrid transgenic immune tolerant mouse model for assessing the breaking of B cell tolerance by human interferon beta. *J Immunol Methods*, *352*, 32–37.
19. Gilli, F., van Beers, M., Marnetto, F., Jiskoot, W., Bertolotto, A., Schellekens, H. (2008). Development of a bioassay for quantification of neutralising antibodies against human interferon-beta in mouse sera. *J Immunol Methods*, *336*, 119–126.

20. Willander, M., Al-Hilli, S. (2009). Analysis of biomolecules using surface plasmons. *Methods Mol Biol*, *544*, 201–229.
21. Aarden, L., Ruuls, S.R., Wolbink, G. (2008). Immunogenicity of anti-tumor necrosis factor antibodies—toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20*, 431–435.
22. Ngai, P.K., Ackermann, F., Wendt, H., Savoca, R., Bosshard, H.R. (1993). Protein A antibody-capture ELISA (PACE): an ELISA format to avoid denaturation of surface-adsorbed antigens. *J Immunol Methods*, *158*, 267–276.
23. Schwab, C., Bosshard, H.R. (1992). Caveats for the use of surface-adsorbed protein antigen to test the specificity of antibodies. *J Immunol Methods*, *147*, 125–134.
24. Schlain, B., et al. (2010). A novel gamma-fitting statistical method for anti-drug antibody assays to establish assay cut points for data with non-normal distribution. *J Immunol Methods*, *352*, 161–168.
25. Bartelds, G.M., Wijbrandts, C.A., Nurmohamed, M.T., Stapel, S., Lems, W.F., Aarden, L., Dijkmans, B.A., Tak, P.P., Wolbink, G.J. (2007). Clinical response to adalimumab: relationship to anti-adalimumab antibodies and serum adalimumab concentrations in rheumatoid arthritis. *Ann Rheum Dis*, *66*, 921–926.
26. Radstake, T.R., Svenson, M., Eijsbouts, A.M., van den Hoogen, F.H.J., Enevold, C., van Riel, P.L.C.M., Bendtzen, K. (2009). Formation of antibodies against infliximab and adalimumab strongly correlates with functional drug levels and clinical responses in rheumatoid arthritis. *Ann Rheum Dis*, *68*, 1739–1745.
27. Yang, J., et al. (2005). Two cases of pure red-cell aplasia due to anti-erythropoietin antibodies. *J Nephrol*, *18*, 102–105.
28. Antonelli, G., et al. (1999). Further study on the specificity and incidence of neutralizing antibodies to interferon (IFN) in relapsing remitting multiple sclerosis patients treated with IFN beta-1a or IFN beta-1b. *J Neurol Sci*, *168*, 131–136.
29. Brickelmaier, M. et al. (1999). ELISA methods for the analysis of antibody responses induced in multiple sclerosis patients treated with recombinant interferon-beta. *J Immunol Methods*, *227*, 121–135.
30. Pachner, A., Narayan, K., Price, N., Hurd, M., Dail, D. (2003). MxA gene expression analysis as an interferon-beta bioactivity measurement in patients with multiple sclerosis and the identification of antibody-mediated decreased bioactivity. *Mol Diagn*, *7*, 17–25.
31. Perini, P., Facchinetti, A., Bulian, P., Massaro, A.R., Pascalis, D.D., Bertolotto, A., Biasi, G., Gallo, P. (2001). Interferon-beta (IFN-beta) antibodies in interferon-beta1a- and interferon-beta1b-treated multiple sclerosis patients. Prevalence, kinetics, cross-reactivity, and factors enhancing interferon-beta immunogenicity in vivo. *Eur Cytokine Netw*, *12*, 56–61.
32. Rudick, R.A., Simonian, N.A., Alam, J.A., Campion, M., Scaramucci, J.O., Jones, W., Coats, M.E., Goodkin, D.E., Weinstock-Guttman, B., Herndon, R.M., Mass, M.K., Richert, J.R., Salazar, A.M., Munschauer, F.E. 3rd, Cookfair, D.L., Simon, J.H., Jacobs, L.D. (1998). Incidence and significance of neutralizing antibodies to interferon beta-1a in multiple sclerosis. Multiple Sclerosis Collaborative Research Group (MSCRG). *Neurology*, *50*, 1266–1272.
33. WHO Expert Committee on Biological Standardisation. (1985). Thirty-Fifth Report. *WHO Technical Report Series*.

34. Files, J.G., Gray, J.L., Do, L.T., Foley, W.P., Gabe, J.D., Nestaas, E., Pungor Jr., E. (1998). A novel sensitive and selective bioassay for human type I interferons. *J Interferon Cytokine Res*, *18*, 1019–1024.
35. Pungor, E. Jr., Files, J.G., Gabe, J.D., Do, L.T., Foley, W.P., et al. (1998). A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. *J Interferon Cytokine Res*, *18*, 1025–1030.
36. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., et al. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321*, 19–31.
37. Sellebjerg, F., Barnes, D., Fillippini, G., Midgard, R., Montalban, X., Rieckman, P., Selmaj, K., Visser, L.H., Sorensen, P.S. (2005). EFNS guideline on treatment of multiple sclerosis relapses: report of an EFNS task force on treatment of multiple sclerosis relapses. *Eur J Neurol*, *12*, 939–946.
38. Bertolotto, A., Malucchi, S., Milano, E., Castello, A., Capobianco, M., Mutani, R. (2000). Interferon beta neutralizing antibodies in multiple sclerosis: neutralizing activity and cross-reactivity with three different preparations. *Immunopharmacology*, *48*, 95–100.
39. Kawade, Y. (1986). Quantitation of neutralization of interferon by antibody. *Methods Enzymol*, *119*, 558–573.
40. Kawade, Y., Finter, N., Grossberg, S.E. (2003). Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. *J Immunol Methods*, *278*, 127–144.
41. Grossberg, L.D., Kawade, Y. (2006). *The Interferons: Characterization and Application*. Wiley-VCH, Weinheim, Germany.
42. Grossberg, S.E., Kawade, Y., Grossberg, L.D. (2009). The neutralization of interferons by antibody. III. The constant antibody bioassay, a highly sensitive quantitative detector of low antibody levels. *J Interferon Cytokine Res*, *29*, 93–104.
43. Damen, C.W., et al. (2009). Development and validation of an enzyme-linked immunosorbent assay for the quantification of trastuzumab in human serum and plasma. *Anal Biochem*, *391*, 114–120.
44. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barret, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quanmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
45. Schellekens, H. (2008). The immunogenicity of therapeutic proteins and the Fabry antibody standardization initiative. *Clin Ther*, *30* (Suppl. B), S50–S51.

18

DETECTION OF ANTIBODIES TO BIOPHARMACEUTICALS IN THE PRESENCE OF HIGH LEVELS OF CIRCULATING DRUG

Arno Kromminga and Michael G. Tovey

18.1. INTRODUCTION

Development of effective and reliable assays for the detection and quantification of anti-drug antibodies (ADAs) depends to a large extent upon the identification of the factors present in samples from patients with the desired disease indication that could potentially interfere with the assay and prevent or distort detection of ADAs present in the sample. Prominent among such factors is the drug itself, or its endogenous counterpart as in the case of a recombinant analogue of a growth factor such as erythropoietin (EPO), or granulocyte–macrophage colony stimulating factor (GM-CSF), or a cytokine such as interferon alpha (IFN-α) or interferon beta (IFN-β). Residual drug, or its endogenous counterpart, will compete with added drug, or the binding reagent to capture drug-specific antibodies, resulting in a lower titer or a false negative result. The presence of residual drug is of particular concern in the case of therapeutic monoclonal antibodies (mAbs) that are administered in high doses and are cleared slowly from the peripheral circulation. As it may not always be feasible to wait until washout of the drug has occurred in order to collect a sample, the level of drug tolerance of the assay should be determined. Accurate quantification of ADA levels is also rendered difficult by

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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the inability to detect ADA bound to drug in the form of soluble immune complexes.

This chapter reviews the relative drug tolerance of different types of ADA assays, together with procedures for determining the drug tolerance of a particular assay. Each assay methodology will be discussed only briefly with respect to its relative drug tolerance since each assay format is described in detail in the other chapters in this volume.

18.2. ASSAY FORMATS FOR DETECTION OF BINDING ANTIBODIES

Assays for the detection of antibodies should provide accurate and precise information about the nature of the antibodies. Several assay formats can be used for the analysis of binding antibodies against protein-based therapeutics. Two principal types of assay format can be used for *in vitro* analysis of antibodies: heterogeneous assay formats in which the target antigen is immobilized on a matrix such as nitrocellulose, for immunoblotting procedures, or on microtiter plates, for ELISA-based assays; and homogeneous assay formats performed in a liquid phase using a soluble target antigen.

18.2.1. Heterogeneous Assay Formats

Initially, the immunogenicity of biologicals was assessed using standard sandwich ELISAs, commonly used as screening assays in clinical diagnostics, for example, for the detection of autoantibodies. Such assays were employed for the detection of antibodies against biologicals. It soon became apparent, however, that these types of assays, in which the antigen is immobilized directly on the surface of the microtiter plate, were subject to misleading results due principally to matrix effects. Nonspecific binding of the antibody to a solid surface, for example, led to false positive results. In addition, masked epitopes not accessible to circulating antibodies, due to the random immobilization of the antigen, led to false negative results. Furthermore, low-affinity antibodies may not be detected due to the harsh washing conditions employed in such assays. Attempts were made to circumvent the disadvantages of direct ELISA formats by using spacers to attach the target antigen to the matrix. In one such approach the target antigen is labeled and captured by using a high-affinity ligand, such as biotin. The biotinylated target protein is then immobilized on pretreated streptavidin-coated plates. Such second-generation ADA assays exhibit improved specificities and sensitivities but are still flawed, due mainly to the use of labeled secondary antibodies (directed against human immunoglobulin isotypes) that restrict their application to the analysis of clinical samples only. In addition, due to the relatively harsh washing conditions employed, low-affinity antibodies may not be deteced. In addition, epitopes may be masked by the use of labeling molecules such as biotin or digoxigenin.

An alternative approach employed to detect binding antibodies is to use bridging ELISAs that obviate some of the disadvantages of standard ELISAs. In this assay format immobilized antigen is used as the capture reagent and a labeled antigen as the detection reagent. Binding antibodies present in patient samples recognize both immobilized and soluble antigen due to their bivalent binding characteristics. The bridging assay has the advantage of being able to detect all immunoglobulin isotypes, since In this format the Fc moiety of the anti-drug antibodies to be detected is not involved in the detection process. Thus, the bridging assay is species independent and can be used for the analysis of samples from both preclinical and clinical studies. It should be noted, however, that a bridging type of ELISA fails to detect monovalent binding antibodies, such as antibodies of the IgG4 isotype.

In both direct and bridging-based ELISAs the detection of anti-drug antibodies is impaired by the limited drug tolerance of the assay. The formation of immune complexes in the presence of high levels of circulating drug leads to reduced assay sensitivity and thus to false negative results. The analytical sensitivity of these types of assays in the absence of residual drug is in the range of 0.5 μg/mL.

Surface plasmon resonance (SPR) technology was originally designed for the characterization of receptor–ligand interactions. In addition to measurement of the concentration of analyte kinetics, SPR can also be used to determine parameters such as association and dissociation constants. This method is also widely used for the characterization of binding anti-drug antibodies (BAbs). An inherent advantage of the system is that it does not rely on the use of a label or secondary detector molecules, and the target antigen can be immobilized directly by covalent coupling to the surface of dextran-coated chips. Alternatively, an indirect procedure can also be employed based on the interaction of biotinylated target antigens with streptavidin-coated chips, or hexahistidine-tagged target proteins bound to Ni-nitrilotriacetic acid (NTA)–treated chips. In all these methods of immobilization a heterogeneous format is used for antibody detection.

In addition to the elimination of the risk of epitope masking due to the use of a label-free format, the broad dynamic range of the SPR technology also permits the detection of low-affinity antibodies. In addition, SPR can also be used to determine immunoglobulin isotype and subclass, providing a more detailed view of the immune response to biopharmaceuticals.

Recently, software has become available for the detection of anti-drug antibodies in the presence of drug, using dissociation of immune complexes by treatment at acidic pH [1].

18.2.2. Homogeneous Assay Formats

The formation of immune complexes involves a steady-state equilibrium, resulting in a constant process of association and dissociation. The addition of a vast excess of labeled target antigen leads to the generation of labeled

immune complexes. Therefore, homogeneous assay formats usually show a higher drug tolerance than heterogeneous assays, unless the tracer and the BABs form immune complexes with high dissociation rates, allowing them to dissociate during the (brief) time it takes to separate bound from free tracer.

The radioimmunoprecipitation assay (RIPA) is a direct liquid-phase assay that detects linear as well as conformational epitopes with high sensitivity. Because the antigen is not immobilized, conformation is not altered and all epitopes are accessible. The RIPA has been used in numerous studies for detection of anti-drug antibodies [2], including antibodies against EPO, IFN-β, G-CSF, and TNF-α antagonists [3, 4]. As the RIPA does not involve the use of a secondary antibody, it is less species dependent and shows a high sensitivity and specificity. On the other hand, the use of protein A or G may lead to a bias in the isotypes detected.

The electrochemiluminescent (ECL) assay provides an alternative nonradioactive homogeneous assay based on the use of both biotin- and ruthenium-labeled antigen. After binding of anti-drug antibodies to both the biotinylated and the ruthenium-labeled drug, the trimolecular complex is immobilized on a streptavidin-coated carbon microtiter plate with embedded electrodes. Application of a voltage potential then activates the ruthenium ion, resulting in light emission that can be quantified. The homogeneous ECL format has a sensitivity and specificity comparable to that of the RIPA and a high dynamic range. It is species independent and shows high drug tolerance due to the homogeneous format previously described. It should be noted, however, that the use of biotin- and ruthenium-labeled antigen introduces the risk of epitope masking.

18.2.3. Detection of Neutralizing Antibodies

18.2.3.1. Cell-Based Assays. Neutralizing antibodies (NAbs) inhibit the biological activity of a biopharmaceutical by binding either to an epitope within or near the active site of the molecule or to an epitope that prevents the drug binding to its cell surface receptor, either directly or indirectly by steric hindrance. This can result in a loss of activity of the drug, necessitating either an increase in the dose administered or cessation of therapy and recourse to alternative therapy, if available. Most biopharmaceuticals function either as agonists or antagonists of growth factors or cytokines; or, as in the case of numerous monoclonal antibodies (mAbs), they interact with a cell surface molecule such as a receptor or integrin. Traditionally, cell-based assays have been used to determine the ability of NAbs to inhibit the biological activity of a drug. A recent white paper defined a cell-based NAb assay as "an *in vitro* assay utilizing cultured cells that interact with or respond to the drug either directly or indirectly in a measurable manner in the presence of test species matrix for the detection of anti-drug product neutralizing antibodies" [5]. Such assays are often based upon a potency assay that measures the ability of the

drug to stimulate or inhibit a particular cellular function, such as proliferation, apoptosis, differentiation, or production of a specific cellular product. These are complex endpoints that involve transcriptional regulation of numerous cellular genes and often require several hours or days before a reading can be taken. Such complex endpoints can often be induced by numerous different factors, thus reducing assay specificity. Endpoints based upon the quantification of the expression of a specific receptor-associated kinase, or drug-induced gene, provide less complex, more rapid, and often more specific assay readouts. If a cell line with suitable attributes is unavailable, then a cell line can be obtained by transfecting cells with a drug-specific receptor and/or a drug-responsive reporter-gene construct, and isolating cells with the desired characteristics. This approach has a number of advantages. Overexpression of the target receptor will increase the signal-to-noise ratio, thereby increasing both assay sensitivity and specificity. The use of a reporter gene also facilitates assay quantification and increases sensitivity and reproducibility. Assay specificity can also be increased by using cells that are naturally receptor negative, or by rendering cells receptor negative, for substances that may interfere with the assay by activating the same signal transduction pathway as the drug of interest.

Determination of the tolerance of a cell-based assay to the presence of residual drug in a sample necessitates the determination of the concentration of drug that when added to the assay will inhibit a NAb positive control and restore drug function. The selection of a positive control is rendered difficult by the polyclonal nature of the human antibody response to treatment with a biopharmaceutical. This results in the production of a mixture of ADAs of varying affinity, avidity, isotype, and epitope specificity, the composition of which changes during the course of treatment. Thus, if suitable human polyclonal positive ADA control preparations are not available, then the concentration of drug that restores full drug activity should be determined for several different monoclonal or polyclonal animal antisera. The use of a high-affinity positive control antibody will yield an apparently low level of drug tolerance, while the use of a low-affinity positive control antibody will result in an apparently high level of drug tolerance. Thus, the apparent level of drug tolerance should be determined for a series of different control ADAs. In practice, drug tolerance is determined by incubating a control ADA sample at the lower limit of quantification (LLOQ) with serial dilutions of drug to determine the concentration of drug that prevents detection of the sample at the LLOQ. This procedure should be repeated for each of the control ADA samples available.

Certain mAbs such as rituximab exert their therapeutic action in part by induction of antibody-dependent cellular cytotoxicity (ADCC) mediated through the Fc moiety of the molecule. The presence of residual drug in a sample can also impact this aspect of the action of certain monoclonal antibodies.

18.2.3.2. Competitive Ligand-Binding Assays. The neutralizing ADA response to therapeutic mAbs often prevents the mAb from binding to its target molecule on the cell surface, thereby preventing the mAb from exerting its therapeutic action. In such cases a competitive ligand-binding assay may often be the assay of choice for the detection and quantification of neutralizing ADAs. Competitive ligand-binding assays are often based on the use of the ECL detection system using the Meso Scale Discovery (MSD, Gaithersberg, MD) analyzer. In this system ADAs are detected through simultaneous binding of the antibody to biotinylated drug and ruthenium-conjugated drug. The ADA and biotinylated drug and ruthenium-conjugated drug complex is then captured on streptavidin-coated carbon plates. A voltage is then applied via electrodes embedded in the carbon plates and light is emitted and quantified. Competitive ligand-binding assays employing the ECL detection system and the MSD analyzer are considerably more drug tolerant than either cell-based NAb assays or immunoassays. Thus, Loyet et al. [6] estimated that an MSD-based assay for ADAs against the TNF-α antagonist Humira could tolerate approximately 1.0 μg/mL of excess Humira (Abbott Laboratories) using a high-titer positive control and approximately 0.25 μg/mL of Humira using a lower-titer positive control [6]. In this study drug tolerance was defined as the maximum amount of drug that yields a signal above the assay cut point [6]. Loyet et al. also determined the drug tolerance of a cell-binding assay using the ECL detection system and the MSD analyzer. This assay was based on Jurkat cells expressing a noncleavable TNF-α mutant to prevent TNF-α being cleaved from the cell surface to produce a soluble form that would interfere with the assay. Loyet et al. [6] showed that this assay for anti-Humira NAbs is highly drug tolerant. Indeed a positive ECL signal above the cut point could be detected even in the presence of some 500 μg/mL of drug compared with a drug tolerance level of approximately 5 μg/mL for a radioimmunoassay for ADAs against Humira [6].

18.3. IMPROVEMENT OF DRUG TOLERANCE BY SAMPLE TREATMENT

Interference due to the presence of residual drug poses a significant challenge for both design and implementation of ADA assays. The presence of high levels of circulating drug results from a combination of the high levels of drug administered and the prolonged half-life of the drug, which in the case of therapeutic mAbs is of the same order of magnitude as the anti-drug antibodies induced in response to treatment. While the serum half-life of endogenous IgG is 21 days, the serum half-life of therapeutic monoclonal antibodies may vary between 9 days for infliximab and up to 62 days for rituximab. Therapeutic monoclonal antibodies are not alone in exhibiting a prolonged serum half-life. Substitution of certain amino acids in erythropoietin has resulted in a prolonged half-life of some 70 hours for darbepoietin, compared with 5 hours for

the unmodified erythropoietin [7]. Similarly, pegylated interferon alpha-2a (PEG-IFN, Pegasys) has a serum half-life approximately 10-fold greater than the 8.5-hour half-life of the unmodified drug [8].

18.3.1. Acid Dissociation of Soluble ADA–Drug Immune Complexes

It is often difficult to accurately quantify ADA levels in the presence of residual drug due to the formation of soluble immune complexes. Dissociation of soluble immune complexes at acid pH followed by neutralization in the presence of solid phase bound antigen provides a means of quantifying drug-bound ADAs sequestered in the form of soluble immune complexes. The exact conditions employed are often critical and should be determined case-by-case. A change of as little as 0.5 pH unit can markedly affect both drug tolerance and signal loss in an immunoassay. In most cases these methods are applicable to immunoassays only [9, 10]. Cell-based assays using drug-induced effects on proliferation, apoptosis, or differentiation, as the endpoints are often not amenable to the use of acid dissociation. Cell-based assays using short-term readouts such as activation of a reporter gene [11] may be more amenable to the use of acid dissociation although this has to be determined case-by-case. An alternative strategy more amenable to use with cell-based assays is to dissociate soluble ADA–drug immune complexes at acid pH and then capture the ADA using ligand, or a suitable capture reagent such as protein A/G, in the case of non-whole-antibody–based therapeutics, bound to a solid surface prior to neutralization of the sample and analysis using the cell-based assay [12].

The most common and widely used step in the treatment of serum samples containing drug and possibly ADA is treatment at acid pH. This leads to a dissociation of the immune complexes, that is, free circulating antibodies that can be measured, after neutralization, by binding to immobilized drug in a direct- or bridging-ELISA format [12]. It should be noted, however, that such treatment can lead to irreversible destruction of the drug.

An alternative approach to increasing the drug tolerance of ADA assays is based upon accumulation of drug-specific antibodies. The novel method, termed affinity capture elution (ACE), has been used for the detection of anti-rituximab antibodies in the presence of rituximab [13, 14]. The authors claim to be able to detect rituximab-specific antibodies at levels of up to a 6:1 ratio of ADA to drug. Moreover, 36 μg/mL of antibodies against rituximab could be detected In the presence of rituximab at levels as high as 64 μg/mL with a recovery rate of 40% [13, 14].

Most recently a method has been described that increases drug tolerance for the detection of antibodies against therapeutic monoclonal antibody adalimumab using acid dissociation followed by addition of an excess of rabbit anti-idiotype-F(ab), which prevented re-formation of immune complexes before neutralization [15]. It was reported that the addition of 2 μg of rabbit F(ab)-anti-idiotype was sufficient to detect ADA in the presence of up to

24 μg/mL of adalimumab in samples of serum from adalimumab-treated patients [15].

18.4. CONCLUSION

Detection of antibodies against therapeutic proteins is often confronted with the challenge of the detection and quantification of ADAs in the presence of a vast excess of the target antigen in marked contrast to the detection of autoantibodies, which is not subject to such constraints [13]. Dissociation of antibody–drug soluble immune complexes at acid pH prior to analysis is one type of approach that can be used for the detection and characterization of ADAs. Moreover, in addition to the detection and quantification of ADAs, determination of the binding properties of the induced polyclonal antibody response against the biopharmaceutical is of considerable interest. Even though anti-drug antibodies can be detected using sensitive methods, their presence may have only minor or no biological consequences. Thus, differentiation of antibodies with respect to their binding and/or neutralizing capacity, using cell-based assays or competitive ligand-binding assays depending upon the type of biopharmaceutical studied, is important for the interpretation of clinical findings.

Analysis of the antibody response induced by the administration of biopharmaceuticals is of considerable medical and economic interest since the presence of certain types of anti-drug antibodies can lead to decreased efficacy of the therapeutic agent, necessitating dose escalation or switching the patient to alternative therapy if available. It is of considerable importance to identify those patients who are no longer able to respond optimally to therapy in order not to subject the patient to treatment with ineffective therapy when alternative therapy may be available. It is also important to obviate unjustified costs to the health care provider. Most important, the patient may be subject to severe and sometimes life-threatening autoimmune disorders in cases where ADAs cross-react with a nonredundant endogenous counterpart, as in the case of erythropoietin [16, 17].

REFERENCES

1. Sickert, D., Kroeger, K., Zickler, C., Chokote, E., Winkler, B., Grenet, J.M., Legay, F., Zaar, A. (2008). Improvement of drug tolerance in immunogenicity testing by acid treatment on Biacore. *J Immunol Methods*, May, *20*, 334, 29–36.
2. Lofgren, J.A., Dhandapani, S., Pennucci, J.J., Abbott, C.M., Mytych, D.T., Kaliyaperumal, A., Swanson, S.J., Mullenix, M.C. (2007). Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *J Immunol*, *178*, 7467.

3. Bendtzen, K. (2010). Critical review: assessment of interferon-beta immunogenicity in multiple sclerosis. *J Interferon Cytokine Res*, *30*, 759–766.
4. Aarden, L., Ruuls, S.R., Wolbink, G., (2008). Immunogenicity of anti-tumor necrosis factor antibodies—toward improved methods of anti-antibody measurement. *Curr Opin Immunol*, *20*, 431–435.
5. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
6. Loyet, K.M., Deng, R., Laing, W.C., Wu, Y., Lowman, H.B., DeForge, L.E. (2009). Technology comparisons for anti-therapeutic antibody and neutralizing antibody assays in the context of an anti-TNF pharmacokinetic study. *J Immunol Methods*, *345*, 17–28.
7. Smith, R. (2002). Applications of darbepoietin-alpha, a novel erythropoiesus-stimulating protein, in oncology. *Curr Opin Hematol*, *9*, 228–33.
8. Foster, G.R. (2010). Pegylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b. *Drugs*, *70*, 147–165.
9. Patton, A., Mullenix, M.C., Swanson, S.J., *and* Koren, E. (2005). An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J Immunol Methods*, *304*, 189.
10. Smith, H.W., Butterfield, A., Sun, D. (2007). Detection of antibodies against therapeutic proteins in the presence of residual therapeutic protein using a solid-phase extraction with acid dissociation (SPEAD) sample treatment prior to ELISA. *Regul Toxicol Pharmacol*, *49*, 230–237.
11. Lallemand, C., Meritet, J.F., Erickson, R., et al. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
12. Lofgren, J.A., Wala, I., Koren, E., Swanson, S.J., Jing, S. (2006). Detection of neutralizing anti-therapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J Immunol Methods*, *308*, 101.
13. Bourdage, J.S., Cook, C.A., Farrington, D.L., Chain, J.S., Konrad, R.J. (2007). An affinity capture elution (ACE) assay for detection of anti-drug antibody to monoclonal antibody therapeutics in the presence of high levels of drug. *J Immunol Methods*, *327*, 10–17.
14. Schmidt, E., Hennig, K., Mengede, C., Zillikens, D., Kromminga, A. (2009). Immunogenicity of rituximab in patients with severe pemphigus. *Clin Immunol*, *132*, 334–341.
15. Van Schouwenburg, P.A., Bartelds, G.M., Hart, M., Aarden, L., Wolbink, G.J., Wouters, D. (2010). A novel method for the detection of antibodies to adalimumab in the presence of drug reveals "hidden" immunogenicity in rheumatoid arthritis patients. *J Immunol Methods*, *327*, 82–88.

16. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.

17. Li, J.,Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.

17.2. EXAMPLE NEUTRALIZING ANTIBODY ASSAY SYSTEM

The fundamental measurement system in a NAb assay is based upon a modified cell culture bioassay that measures the antigen in samples. The goal of the modification is to make the assay measure the activity of antibodies in a specimen that neutralize the antigen. The modified bioassay may fix the concentration of either the antigen or the sample in order to yield a functional dilution–response curve [2].

Nearly all NAb assay systems are based on a family of four-parameter logistic dilution–response curves [3]:

$$y = \frac{A}{1+\exp[-B(\log(\text{concentration})-C)]} + D + \varepsilon \tag{17.1}$$

where A is the range, B is the slope, C is $\log(\text{ED}_{50})$, and D is the lower asymptote (for illustration, see Fig. 17.1). Both measurement bias and variation around the dilution–response curve are inherent properties of the assay. The usual strategy for designing a NAb assay is to choose a dynamic range and a

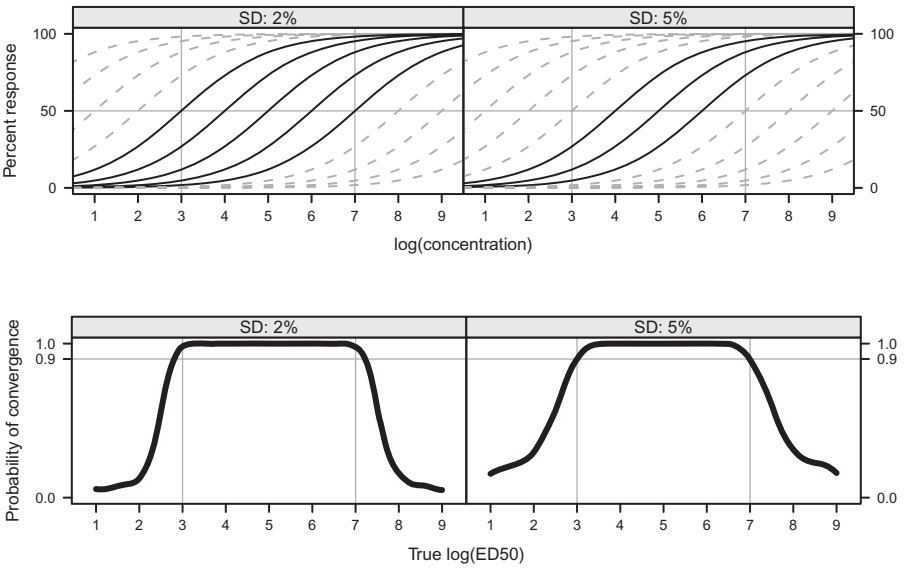

Fig. 17.1 (Upper panels) Four-parameter logistic curves fit to simulated data from designs having 11 equally spaced levels of log(concentration) and either low or moderate variation around the curve (left versus right panel); the solid curves had at least a 95% probability of convergence; the range of ED_{50} values with likely convergence narrows with increased variation around the curve. (Lower panels) The probability of convergence drops for values of $\log(\text{ED}_{50})$ far from the center of the dilution range; the drop starts further from the center but is steeper when there is lower variation around the curve.

replication scheme that yield acceptable precision and bias for the intended use of the assay. The response measure is generally a titer; however, sample-specific values of $\log(ED_{50})$ are preferable because comparisons among them have low variance and they are invariant to slope for curves having similar asymptotes.

The usual approach to calibration, validation, and monitoring of bioassays is to use samples having known values to measure bias. Because a NAb assay is intended to be used on samples from real subjects, it is difficult to create representative samples that have known values. Instead, it is practical to construct a small set of samples for the assay from a specimen drawn from a single subject; the relative concentrations of spiked samples from the same specimen are known. Assuming a single specimen has high NAb response and sufficient volume, the amount of information about dilutional linearity available from that specimen is limited by the dynamic range of the assay. If the within-specimen dilution bias is small and consistent across specimens, then the assay can be said to have good dilutional linearity.

17.3. MEASUREMENT AND TRUNCATION BIAS

Bias in the measurement of $\log(ED_{50})$ increases as the dilution–response curve has more incomplete data on an asymptote (such as in Fig. 17.1, grey dashed lines). The impact of missing information increases substantially with additional variation around the curve; however, it decreases slightly with additional dilutions in the design (Fig. 17.2). For dilution–response curves where high concentration corresponds to high response (proliferation curves as in Fig. 17.1), measurement bias is low for low values of ED_{50} and high for high values (Fig. 17.2, top panel). The potential dynamic range of the assay is the region where the curve fit has high probability of convergence and the $\log(ED_{50})$ has low bias and standard deviation due to measurement errors as well as low trend in measurement bias across the dynamic range (Fig. 17.2).

Truncation bias arises when estimated ED_{50} values are restricted to be within the dynamic range of the assay. Near the boundaries of the dynamic range, estimates of ED_{50} that are more extreme than the true value are eliminated from the data set used for analysis, leaving less extreme values from which to assess dilutional linearity. Truncation bias therefore yields high estimates for low values of ED_{50}, and low estimates for high values of ED_{50}. The magnitude of truncation bias increases with increased variation around the curve.

Measurement and truncation error are not estimable; however, under certain assumptions (similarity) we can predict the amount of measurement bias via simulation based upon the properties of the assay (variation around the curve, shape of the curve, and dilution design; see Fig. 17.2). For proliferation curve shapes as in Figure 17.1, the estimated dilution bias [estimated slope to $\log_2(1/\text{spike})$] will be positively affected by measurement bias; however, it

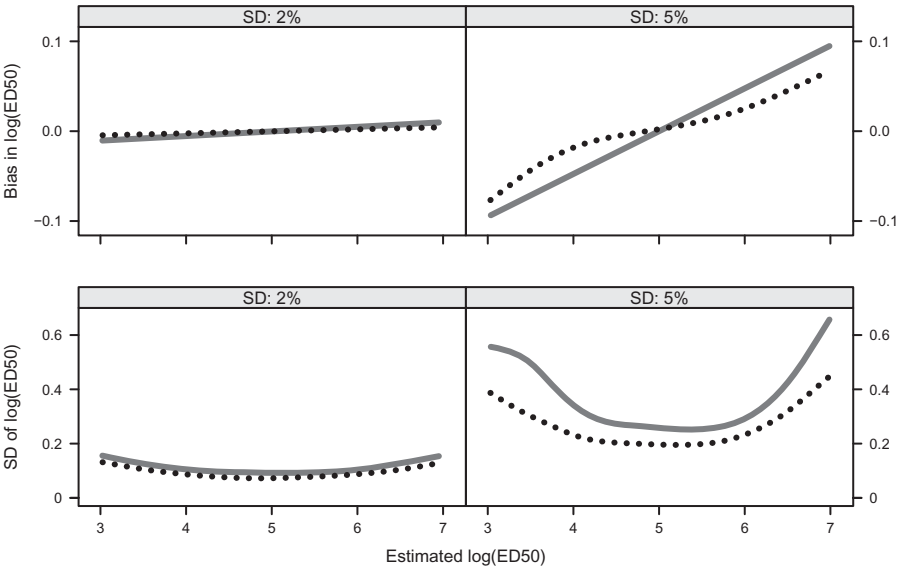

Fig. 17.2 Average measurement bias (top panels) and standard deviation of estimated $\log(ED_{50})$ (bottom panels) from simulated data sets having either low or moderate variation around the dilution–response curve (left versus right panel) and either 7 or 11 equally spaced dilutions (solid versus dashed lines). The bias trend is monotone across the range of estimated $\log(ED_{50})$ values and is in the same direction as the slope of the dilution–response curves (see Fig. 17.1); the variation in estimated $\log(ED_{50})$ increases as the curve becomes more off-center with respect to the dilution range. The impact of measurement error is greater for curves having more variation in response (right panels) and for designs having fewer points (solid lines).

is more strongly negatively affected by truncation bias. (While model-based correction for measurement bias is feasible, in this example it tends to exacerbate the bias due to truncation.)

17.4. DILUTIONAL LINEARITY

The goal of a dilutional linearity study is to use data from samples having ED_{50} above the dynamic range to show that the estimated bias trend is consistent across samples. To estimate dilutional linearity for each specimen, samples are prepared with some replication at each of several spike dilutions spread at known intervals across the range of the assay. Each of these spiked samples yields an entire dilution–response curve, and a summary measure (such as ED_{50} or titer) is estimated from these data.

To assess dilutional linearity, we fit a model to the estimated ED_{50} values for the replicated spiked samples from many specimens. This linear mixed

effects model simultaneously accounts for overall dilution bias trend (population bias) and specimen-specific dilution bias:

$$\log_2(\mathrm{ED}_{50} \times \mathrm{spike}) = \alpha_{\mathrm{specimen}} + (\beta + b_{\mathrm{specimen}}) \log(1/\mathrm{spike}) + \varepsilon \qquad (17.2)$$

where $\alpha_{\mathrm{specimen}}$ is the $\log_2(\mathrm{ED}_{50})$ for a neat sample, fixed effect β is the population dilution bias for a twofold increase in spike dilution, and random effect $b_{\mathrm{specimen}} \sim N(0,\sigma_b^2)$ is the specimen-specific dilution bias for a twofold increase in spike dilution. In the linear mixed effects model (Eq. 17.2), the slope to spike dilution is estimated for each specimen; a slope of zero corresponds to perfect dilutional linearity (note that the response in this model is effectively a spike-dilution-corrected estimate of neat ED_{50}), whereas a nonzero estimate of β corresponds to population dilution bias (Fig. 17.3, top panel), and a large

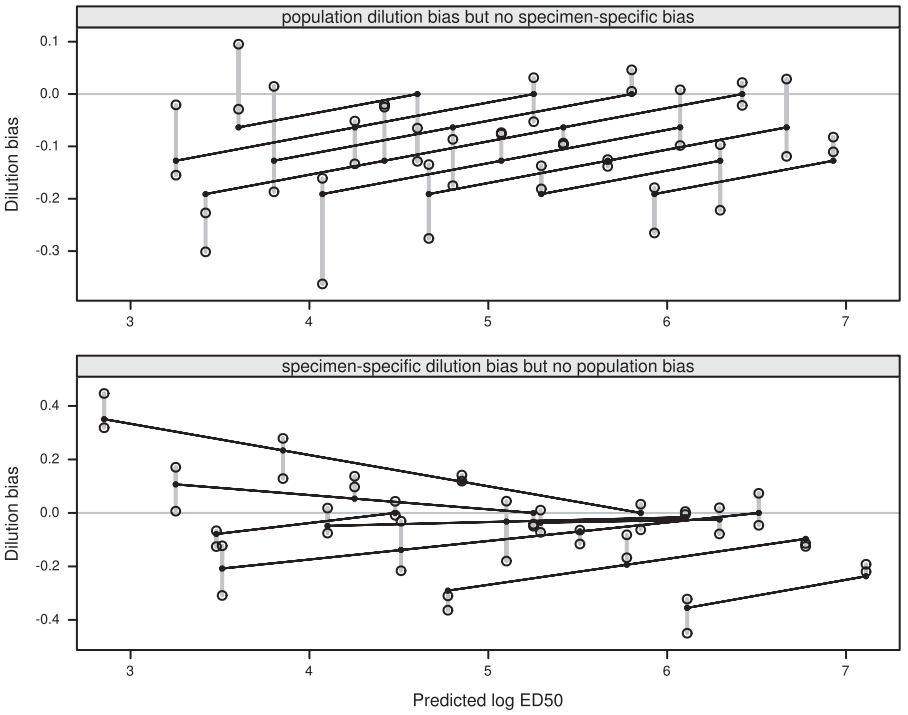

Fig. 17.3 Specimen-specific dilutional linearity for two distinct scenarios: a consistent overall trend in dilution bias for all specimens but no variation in bias among specimens (upper panel), and variation in bias among specimens but no systematic overall trend in bias (lower panel). The design used two samples at each of four spike dilutions for each specimen. The open circles represent observed bias in $\log(\mathrm{ED}_{50})$ (y-axis) versus predicted $\log(\mathrm{ED}_{50})$ under an assumption of no dilution bias (x-axis). The solid points are specimen-specific predictions from a dilutional linearity model (Eq. 2) fitted to samples from specimens having ED_{50} estimates from at least two spike dilutions within the dynamic range of the assay.

estimated standard deviation of the random effects (σ_b) corresponds to specimen-specific dilution bias (Fig. 17.3, bottom panel).

17.5. SIMULATIONS AND MODEL PERFORMANCE

The performance of the linear mixed effects model (Eq. 17.2) was assessed under a variety of simulated scenarios and designs. The scenarios included all combinations of the following conditions: low or moderate variation around the response (SD = 2% or 5% of the response range), population dilution bias of 0% or 10% of the response range, and specimen-specific variation in dilution bias of 0% or 10% of the response range. The designs used each of {2, 3, 4, 5} spikes per specimen combined with each of {sqrt(2), 2, 4, 8}-fold spacing between spikes and either a 7-point or an 11-point dilution–response curve. For the sake of simplicity, the simulations were based upon four-parameter logistic curves with similarity among specimens and constant normal variance around the curves; in practice the constant-variance assumption is reasonable only after a suitable transformation or rescaling of the response.

Estimated ED_{50} values were determined for each sample replicate based upon fitting the logistic model (Eq. 17.1) to each 7-point or 11-point dilution series. The data set for the dilutional linearity assessment associated with each scenario and design combination comprised collections of estimated ED_{50} values for all of the spiked samples (2 replicates per spike) associated with 60 specimens having neat ED_{50} values spread across the dilution range of the assay. Based upon the model convergence performance depicted in Figure 17.1 (bottom panels), the data for each assessment were restricted to a dynamic range of $3 \leq \log_2(ED_{50}) \leq 7$, and only specimens which were represented by at least two spike dilutions after restricting to this dynamic range were used in the analyses (see Fig. 17.3).

The results of these simulations are depicted in Figure 17.4, where each point corresponds to the average estimated bias (or pooled SD) across 50 simulated data sets. For low variation in the response (SD = 2%) the model performs well, adequately recovering the nominal dilution bias and SD values for all scenarios. The model performs best for designs where the spiked samples for each specimen cover the largest possible proportion of the dynamic range of the assay (i.e., 5 spiked samples per specimen with sqrt(2)-fold spacing between spikes, or 2 spiked samples per specimen with 8-fold spacing between spikes).

For moderate variation in the response (SD = 5%), the performance of the model suffers: the estimated population bias tends to be biased low regardless of the nominal amount of population bias (Fig. 17.4, top panels, second and fourth columns), and the estimated variation in specimen-specific bias is higher than the nominal amount (Fig. 17.4, bottom panels, second and fourth columns). It is suspected that this poor performance of the model is due to the heavy impact of truncation bias after only moderate increase in variation in the response. Truncation bias is also implicated in the specimen-specific estimates of slope (Fig. 17.5), which tend to be biased low (also corresponding to low

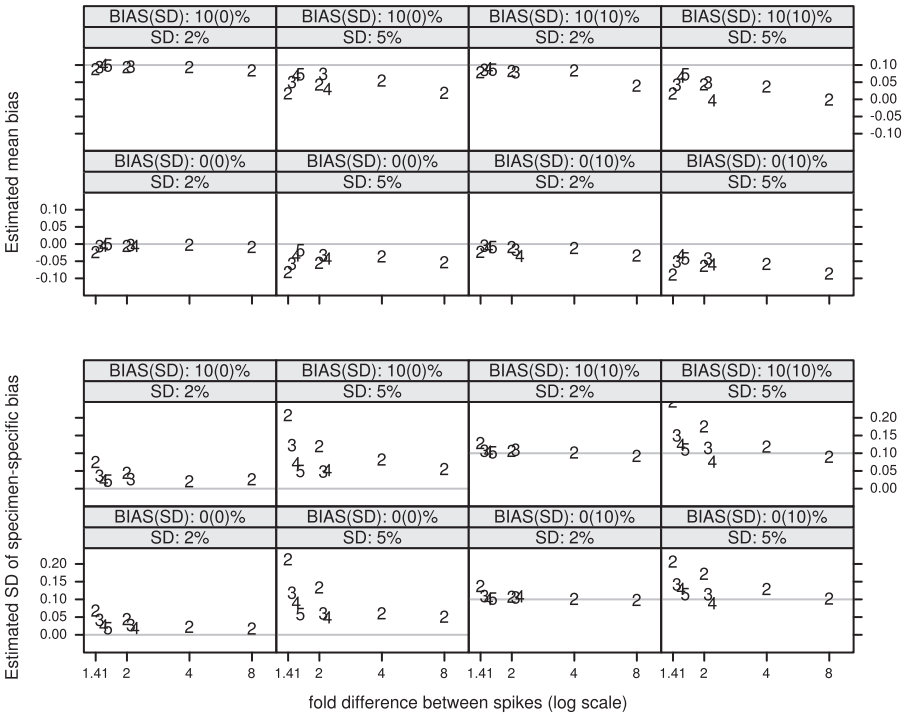

Fig. 17.4 Simulation results: average recovery of nominal amounts of population bias and variation in specimen-specific bias under a variety of simulated scenarios (across panels) and designs (within panels). The scenarios included all combinations of the following conditions: low or moderate variation around the response (SD = 2% or 5%), population dilution bias of 0% or 10%, and specimen-specific variation in dilution bias of 0% or 10%. The designs used 2–5 spikes per specimen (denoted as plotting symbols) with sqrt(2)-fold through 8-fold spacing between spikes (x-axis) and an 11-point dilution–response curve. Each point corresponds to the average (or pooled SD) of estimates from 50 models for each scenario and design. The horizontal line in each panel corresponds to the nominal value of the estimate for the given scenario.

probability of obtaining an estimate) for specimens having values of neat ED_{50} near the boundary of the dynamic range. Additional simulations using larger values (50%) for population dilution bias or variation in specimen-specific dilution bias produced results similar to those in Figure 17.4 (data not shown) where truncation bias remained suspect in causing poor recovery of nominal simulation values.

17.6. DISCUSSION

Demonstrating dilutional linearity of a NAb assay involves properties of the dilution–response assay as well as properties of the population from which specimens will be drawn. Since representative specimens or samples with

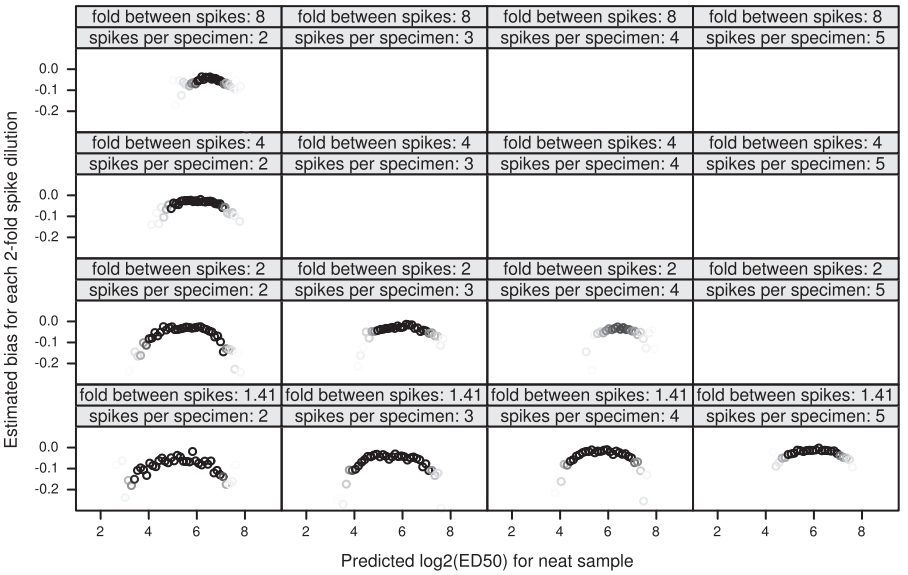

Fig. 17.5 Average estimated specimen-specific slope to $\log_2(1/\text{spike})$ for designs having various numbers of spikes per specimen and fold differences between spikes. Sixty specimens were simulated to have neat-ED_{50} values that spanned the dilution range of the assay. The scenario depicted here had no population or specimen-specific dilution bias and used 11-point dilution–response curve with moderate (5%) variation around the curve. The grey-level of the open circles is proportional to the probability of obtaining a random effects estimate for the slope of the specimen; the bias evident with low probabilities is likely due to truncation to samples that had estimated ED_{50} values within the dynamic range. For a similar figure having low variation around the curve (SD = 2%, not shown), the estimated bias within each panel is markedly less curved.

known amounts of NAb activity are not available, it is necessary to rely upon known relative concentrations of within-specimen spike dilutions to assess dilutional linearity. Using good mixed model design and analysis (15 or more specimens, each with several spiked samples and, for most specimens, a within-specimen spike range that spans most of the dynamic range of the assay) supports estimation of population dilutional bias and variation in specimen-specific dilution bias, but requires a high precision dilution–response assay; substantially more sampling and assay replication may be needed to achieve adequate precision. In a validation study that is intended to demonstrate dilutional linearity of a NAb assay, acceptance criteria should be framed as equivalence tests that demonstrate that the population dilution bias and variation in dilution bias among specimens are small enough for the intended population and use of the assay [1].

For some intended uses of NAb assays, certain types of departures from perfect dilutional linearity are acceptable. Because sample order (on average)

is preserved in the presence of population bias in dilutional linearity, population bias may be acceptable for NAb assays used to compare specimens or establish relationships between NAbs and other biological responses. Similarly, NAb assays with certain systematic patterns of variation in specimen-specific slope to spike dilution (i.e., consistently low bias for low and moderate ED_{50} but increasing negative bias for high ED_{50}), may be completely adequate for certain purposes. On the other hand, if there is appreciable random variation in specimen-specific slope to spike dilution, estimates of NAb activity no longer have a consistent relationship to the true value of NAb activity and the assay may have limited value.

Promising areas for future research include:

- Alternate approaches to formalize and measure departures from dilutional linearity.
- Practical methods to assess, estimate, or control truncation bias, possibly through design.
- Methods to recast NAb assays to include a relevant reference. These might allow assessment of similarity (potentially detecting samples for which the assay is not suitable) and estimation of potency (which can be expected to have higher precision than estimates of ED_{50}).

REFERENCES

1. United States Pharmacopeia. (2009). Biological assay validation. *Pharmacopeial Forum*, **35** (2), March–April (chap. <1033>).
2. Kawade, Y., Finter, N., Grossberg, S.E. (2003). Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affnity. *J Immunol Methods*, **278**, 127–144.
3. Ratkowsky, D.A., Reedy, T.J. (1986). Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. *Biometrics*, **42**, 575–582.

16

CUT POINTS AND PERFORMANCE CHARACTERISTICS FOR ANTI-DRUG ANTIBODY ASSAYS

Viswanath Devanarayan and Michael G. Tovey

16.1. INTRODUCTION

Administration of therapeutic proteins, including recombinant homologues of authentic human proteins, can result in a loss of immune tolerance, leading to an immune response and the production of anti-drug antibodies (ADAs). Although this may be without any apparent untoward consequences for the patient, in some cases ADAs can severely impair the safety and efficacy of therapeutic proteins, leading to reduced pharmacokinetics, pharmacodynamics, bioavailability, and efficacy. ADAs can also cause immune complex disease, allergic reactions, and in some cases severe autoimmune reactions [1, 2]. Assessment of immunogenicity is thus an important part of drug safety evaluation, in both preclinical and clinical studies, and is a prerequisite for the development of less immunogenic and safer biopharmaceuticals. Monitoring patients for the presence of ADAs, as well as the correlation of immunogenicity with clinical data, is therefore key for determining the safety of treatment and for the interpretation of clinical data.

A prerequisite for obtaining reliable immunogenicity data is the development of assays, with appropriate performance characteristics, for the quantification of ADAs. Validation of an immunogenicity assay requires careful evaluation of various characteristics such as sensitivity, low positive control, both biological and analytical variability, and in-study acceptance criteria that

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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will be outlined in this chapter. Most notably, these include determination of the screening-assay cut point, above which a sample is potentially positive (reactive) and below which the sample is most probably negative, as well as establishment of the cut point for confirmatory assays. Samples that are above the screening cut point are tested in a confirmatory assay to determine whether the observed ADA levels are specific to the study drug. For this purpose, a confirmatory cut point is determined from drug-spiked negative samples.

Recommendations on the design and testing strategy for immunogenicity assays have been published in white papers [3–5]. It is assumed for the purposes of this chapter that the assay format and design have been finalized. The estimation of dilution linearity for a neutralizing antibody (NAb) assay is described in detail in Chapter 17, by Lansky and Wager, and will not be discussed further in this chapter. The reader is also referred to the article by Shankar et al. [5] for a detailed description of many of the topics addressed in this chapter.

16.2. MINIMUM REQUIRED DILUTION

The minimum required dilution (MRD) should be determined prior to initiation of the validation experiments. The MRD is established using a series of 5–10 individual samples, tested at dilutions ranging typically from 1:5 to 1:100. The MRD is often determined from the mean reading at each dilution divided by the reading obtained from the reagent blank. It is preferable, however, to determine the signal-to-background value using the Z'-factor, which takes into consideration both the intensity of the assay readout and variability of the readings obtained at different dilutions. The Z'-factor is obtained from the formula

$$\frac{[\text{mean}(S) - 3\text{SD}(S)] - [\text{mean}(B) + 3\text{SD}(B)]}{\text{mean}(S) - \text{mean}(B)}$$

where S is the assay readout, B is the reading obtained for the reagent blank, and SD is standard deviation. Thus, the MRD is the dilution that results in a desired value for the Z' factor and signal-to-background ratio. This metric is widely used for high-throughput screening assays to ensure adequate confidence in the ability to discriminate between signal and background.

16.3. BALANCED DESIGN FOR CUT-POINT DETERMINATIONS

It is important to use a balanced assay design when determining the assay cut point. The design should be balanced in terms of both sample sets and plates analyzed by different analysts. Examples of an unbalanced and a balanced design are shown in Table 16.1. In the assay design shown in Table 16.1A not

TABLE 16.1A Balanced versus Unbalanced Designs for Determination of All Cut Points: Not All Samples Tested in Every Run

			Validation Serum Samples		
Analyst	Assay Run	Assay Plate	$S_1 - S_{16}$	$S_{17} - S_{32}$	$S_{33} - S_{48}$
A_1	R_1	P_1	X		
		P_2	X		
		P_3	X		
	R_2	P_1		X	
		P_2		X	
		P_3		X	
	R_3	P_1			X
		P_2			X
		P_3			X
A_2	R_4	P_1	X		
		P_2	X		
		P_3	X		
	R_5	P_1		X	
		P_2		X	
		P_3		X	
	R_6	P_1			X
		P_2			X
		P_3			X

The experimental layout illustrated in Table 16.1A–C depicts an assay design for a screening-cut-point determination with 48 samples (S1–S48) assayed in three runs (R1–R3) by each of two analysts (A1, A2) with three plates per run (P1–P3). Table 16.1A illustrates an unbalanced layout in which differences between runs are confounded with differences between sample sets, while Table 16.1B illustrates another unbalanced layout in which differences between analysts are confounded with differences between sample sets. Table 16.1C illustrates a balanced layout in which every sample is tested on every run, on every plate, and by both analysts.

all samples are tested in every run, and hence the differences between runs are confounded with differences between the sample sets; in Table 16.1B not all the samples are tested by the two analysts, and hence differences between analysts are confounded with differences between sample sets. These imbalances are corrected in the assay design shown in Table 16.1C such that all samples are tested in every run by both analysts.

16.4. SCREENING CUT POINT

It is useful to establish a flowchart for a screening-cut-point evaluation similar to that shown in Figure 16.1 to ensure that a rational step-by-step procedure is employed. When determining the cut point for a screening assay above

TABLE 16.1B Balanced versus Unbalanced Designs for Determination of All Cut Points: Not All Samples Tested by the Two Analysts

			Validation Serum Samples			
Analyst	Assay Run	Assay Plate	$S_1 - S_{12}$	$S_{13} - S_{24}$	$S_{25} - S_{36}$	$S_{37} - S_{48}$
A_1	R_1	P_1	X			
		P_2	X			
		P_3	X			
	R_2	P_1		X		
		P_2		X		
		P_3		X		
	R_3	P_1			X	
		P_2			X	
		P_3			X	
A_2	R_4	P_1		X		
		P_2		X		
		P_3		X		
	R_5	P_1			X	
		P_2			X	
		P_3			X	
	R_6	P_1				X
		P_2				X
		P_3				X

which a sample is potentially positive (reactive) and below which the sample is considered negative, it is preferable that the cut point should be set such that there is a trend toward false positives rather than false negatives when samples are subsequently reanalyzed in a confirmatory assay. The appropriate cut point for a screening assay should be established during prestudy assay validation from the analysis of representative samples from a drug-naive target patient population, to avoid post hoc data-driven analysis introducing bias into the results. It is important that an adequate number of individual samples, usually 50, are used to establish the cut point. The use of pooled sera should be avoided since this will provide data on analytical variation rather than biological variation. In cases where it is not feasible to obtain serum samples from the target disease population, sera from healthy drug-naive subjects are often employed. Once samples from the target disease population become available the cut-point data should be re-evaluated. If the means and variance of the results from the two populations are comparable, then the original cut point can be retained; otherwise the cut point determined in the target disease population should be used.

TABLE 16.1C Balanced versus Unbalanced Designs for Determination of All Cut Points: All Samples Tested Every Run by Both Analysts

			Validation Serum Samples		
Analyst	Assay Run	Assay Plate	$S_1 - S_{16}$	$S_{17} - S_{32}$	$S_{33} - S_{48}$
A_1	R_1	P_1	X		
		P_2		X	
		P_3			X
	R_2	P_1		X	
		P_2			X
		P_3	X		
	R_3	P_1			X
		P_2	X		
		P_3		X	
A_2	R_4	P_1	X		
		P_2		X	
		P_3			X
	R_5	P_1		X	
		P_2			X
		P_3	X		
	R_6	P_1			X
		P_2	X		
		P_3		X	

16.4.1. Flow Scheme for the Evaluation of the Cut Point for a Screening Assay

Approximately 50 individual sera from drug-naive individuals, preferably from the target population, are assayed in six runs—by two to three analysts if multiple analysts are to be involved in testing the study samples. The distributions of the original and log-transformed data are then evaluated using the Shapiro–Wilk test and the skewness coefficient. The scale (original or logarithmic) that most closely approximates to normal or is most symmetric should be used in all subsequent analyses of the data, such as for outlier evaluation, cut-point calculations, and comparisons of means and variances across assay runs (see Figure 16.4).

Outliers should then be identified and the distribution of the data evaluated after removing the outliers. If the Shapiro–Wilk test is not significant and/or the skewness coefficient is less than 1, the parametric approach (mean + 1.645 × SD) can then be used for determining the cut point from these validation data (CP.V). This will yield a 5% false positive rate from a

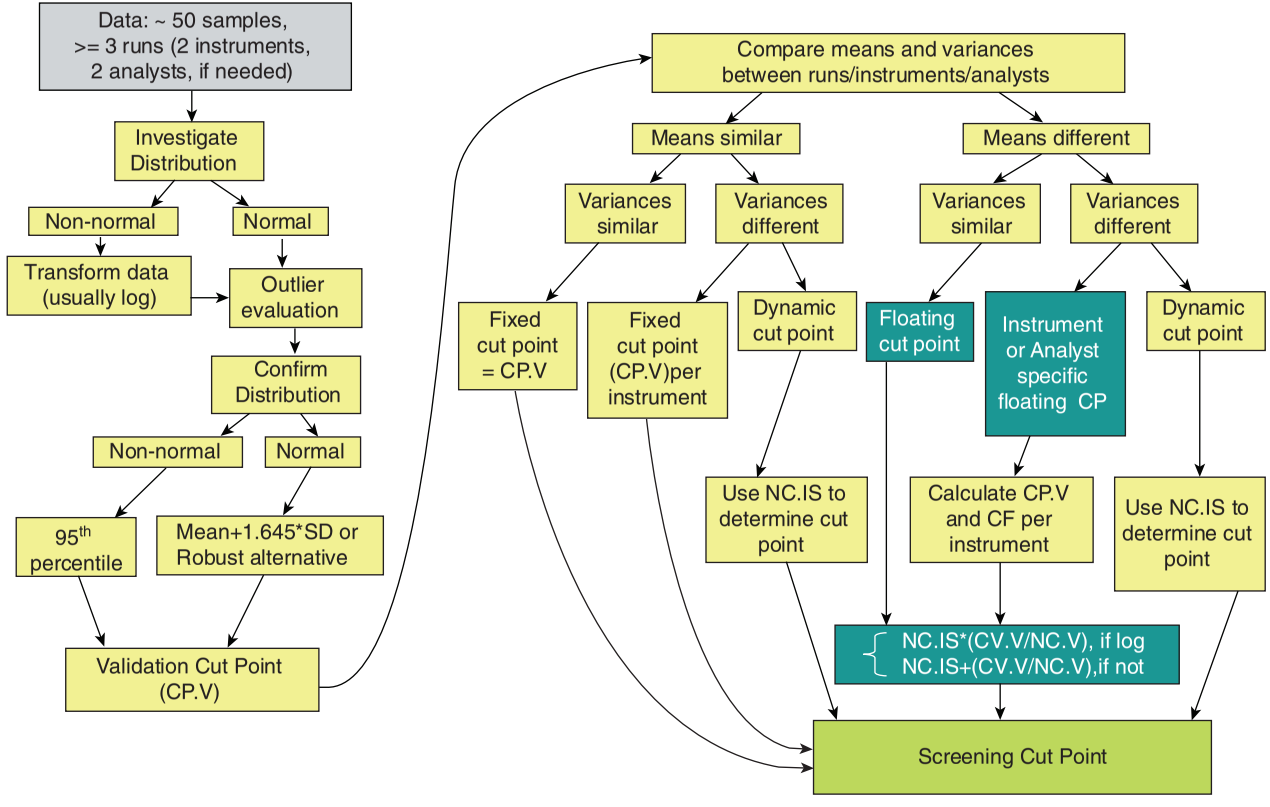


Fig 16.1 Flow scheme for screening-cut-point evaluation (NC.V = negative control from validation runs; NC.IS = negative control from in-study run). (See color insert.)

normal distribution. Otherwise, the nonparametric 95th-percentile approach is recommended. This nonparametric approach should be used as a last resort because it typically requires a much larger number of samples (>100) in order to obtain a reliable result.

The means and variances of the data should then be compared between the assay runs. If both the means and variances are not significantly different, then the same cut point determined from these validation data (CP.V) can be used as the screening cut point; such a screening cut point is called a **fixed cut point**.

If the assay run means alone are significantly different (Fig. 16.2), then a separate screening cut point should be determined for each plate or run during the in-study phase. For this purpose, a correction factor (CF) should be determined from these validation data (data from this screening-cut-point experiment). This is simply the ratio of CP.V to the negative control used in determining the screening cut point (NC.V), or it is the difference between CP.V and NC.V; the former is called the multiplicative correction factor (MCF) and the latter the additive correction factor (ACF). The screening cut point is then defined either by multiplying the negative control reading from each plate determined during the screening phase (NC.IS) by MCF if log-transformation was used in the analysis, or by adding NC.IS to ACF if the analysis was carried out using the original scale. This type of screening cut point is called the **floating cut point**, which from our experience is the most frequently used method for determining the screening cut point.

If the assay run variances differ significantly (Fig. 16.3), and if this is due to differences between analysts or instruments, then a separate floating cut point should be evaluated as just described, for each analyst or instrument. In some

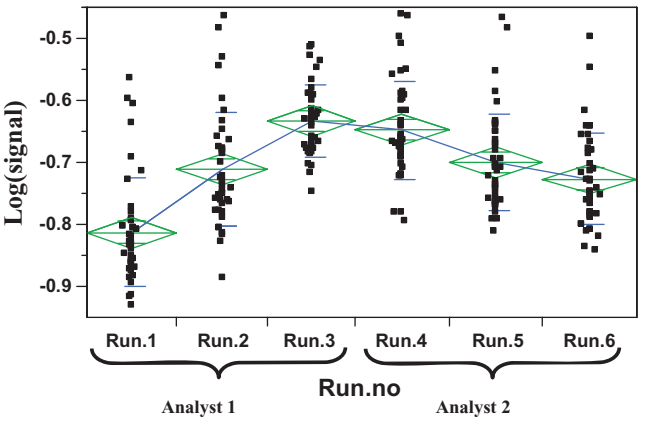


Fig. 16.2 Comparison of means and variances between assay runs; assay run means are significantly different ($p < 0.0001$) and variability is not significantly different (Levene's test, $p = 0.37$), thus a floating-cut-point method should be used. (See color insert.)

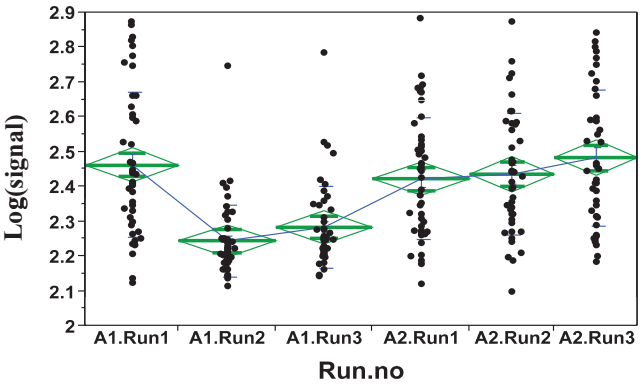


Fig. 16.3 In the axis labels, A1 and A1 refer to the two analysts, and Run1, Run2, and Run3 refer to the three runs performed by each of these two analysts. The means are significantly different ($p < 0.0001$) and variability is significantly different (Levene's test, $p < 0.001$), thus the data suggest using a dynamic cut-point; however, a floating cut point can be used in some cases (see text for details). (See color insert.)

cases assays can be reoptimized to resolve unequal variability between assay runs. If this is not possible and if the reasons for the difference in variance are unknown, then the screening cut point should be evaluated separately for each run by using data from at least 10 individual drug-naive negative sera in every assay run of the in-study phase. After evaluating the distribution and outliers as just described, the screening cut point can be based on the parametric method using the same scale as that employed to obtain the data. This type of screening cut point is called the **dynamic cut point**. This evaluation is often not feasible from a practical perspective, especially when using 96-well instead of 384-well plates. So a compromise may be made by pooling the variability across the six assay runs and using the floating-cut-point method to define the screening cut point in the in-study phase.

We now provide a little more detail on some of the steps involved in the screening-cut-point evaluation.

16.4.2. Identification of Outliers

A simple method for identifying outliers is to use the "outlier box-plot" criteria on the distribution of data (Fig. 16.4). According to these criteria, samples that fall above $Q3 + 1.5 \times (Q3 - Q1)$ or below $Q1 - 1.5 \times (Q3 - Q1)$ are considered as statistical outliers. Here Q3 and Q1 refer to the third quartile (75th percentile) and first quartile (25th percentile), respectively, of the distribution of data.

16.4.3. Analytical versus Biological Outliers

The outlier box-plot criteria described in Section 16.4.2 should be used to analyze the distribution of data from each assay run and to analyze the distri-

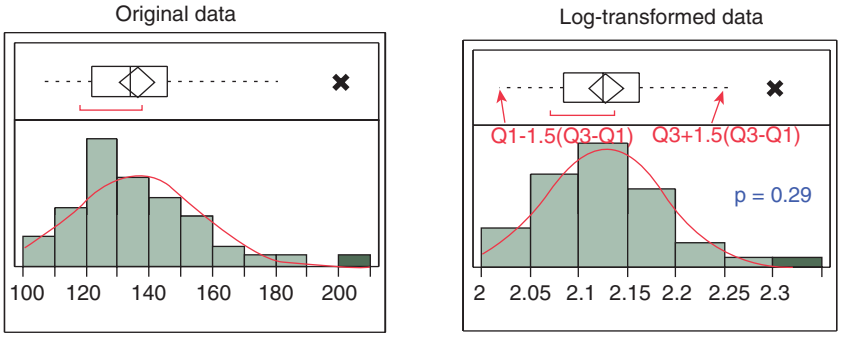


Fig. 16.4 Evaluation of distribution and outliers for data from screening-cut-point experiment (distribution of data from 48 subjects, averaged from 6 runs). Log-transformed data are relatively less non-normal. Subsequent analysis is therefore conducted in log-scale. This also illustrates the criteria for identifying outliers.

bution of averaged results across the six assay runs. Outliers from the averaged data are called **biological outliers** because these are subjects that consistently yield unusually high or low results; that is, most of the samples from these subjects are outliers. All the other outlier samples identified from the distribution of each assay run are considered as **analytical outliers**. These are samples from subjects that did not consistently yield outlier samples across the six assay runs, suggesting that the most likely cause for such outlier samples may be specific analytical errors encountered while assaying such samples.

16.4.4. The 3 × SD Criterion for Identifying Outliers

A popular approach for identifying outliers is to look for samples for which readings fall outside the 3 × SD limits of the distribution of the data, where SD is the standard deviation. Use of this method is not recommended because the estimate of SD is significantly inflated and biased by the presence of outliers, such that the very purpose of identifying outliers is defeated by the use of this approach. To identify outliers, it is critical to use a method that is not influenced by the presence of outliers, and the outlier box-plot method described in Section 16.4.2 is one such robust approach. If, however, the 3 × SD idea is preferred over the outlier box-plot method, an alterative estimate of SD that is less influenced by the presence of outliers is to multiply the median absolute deviation (MAD) by 1.482, in which case the 3 × SD rule is determined by evaluating 3 × 1.482 × MAD.

16.4.5. Low Outliers

Outliers in the lower extreme of the data distribution are sometimes included in the calculation of the screening cut point because it is thought that these

outliers will result in underestimation of the screening cut point, and thus when employing a risk-based approach it is acceptable to err on the side of positives. This argument is flawed, however, because inclusion of the low outliers can cause a significant overestimate of the standard deviation. Thus, while the mean is underestimated, overestimation of the SD can result in a higher screening cut point, thus compromising the 5% false positive rate. It is recommended, therefore, that equal consideration should be given for both the low outliers and high outliers.

16.4.6. Comparisons of Data between Assay Runs, Analysts, and Other Factors

The means between assay runs can be compared using a mixed effects model with runs as the fixed effect and subjects as the random effect. To test for the difference in the results obtained between analysts, a similar mixed effects model can be used with the analyst as the fixed effect, assay runs nested within the analyst, and subjects as random effects. The variances between assay runs can be compared using Bartlett's test or Levene's test.

16.4.7. Evaluation of the Standard Deviation for Cut-Point Calculations

It is essential to apply appropriate methods that consider both the analytical intra-assay variability and the biological intersubject variability for evaluation of the standard deviation when the parametric method is used to determine the screening cut point. A method that is commonly employed is to average the results obtained for six assay runs from each subject, and to determine the standard deviation of these averaged results. This approach is not recommended because it considers only the biological variability and ignores analytical variability. A simple and more correct alternative is to evaluate the variance of the subject samples from each assay run, and then to use the square root of the average of these variances to calculate SD. If there are no outliers in the data, then the average can be determined as a simple arithmetic mean of the variances from each assay run; otherwise, a weighted average should be used, where the weight for each assay run is simply the number of nonoutlier samples from that run minus 1. This approach is easy to implement in a spreadsheet application, and it considers both the intra-run analytical variability and the inter-subject biological variability. A more statistically rigorous method to evaluate SD would be to employ a random effects model with assay runs and subjects as random effects, and to use the variance component estimates corresponding to the subject term (inter-subject variance) and the residual term (intra-assay variance) from this model.

16.4.8. Validity of Negative Controls for Determination of a Floating Cut Point

An assumption made in the floating-cut-point approach is that the negative control during the in-study phase will vary in the same direction as the indi-

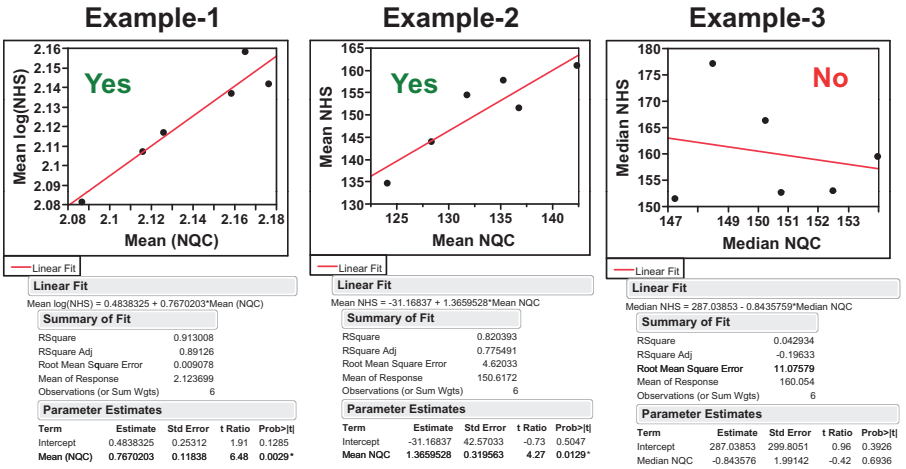


Fig. 16.5 Correlation of negative control versus average of subject samples from six runs of three screening-cut-point experiments. In Examples 1 and 2, R^2 values of 0.91 and 0.82, respectively, and significant linear regression slopes ($p = 0.0029$ and 0.0129, respectively) indicate adequate correlation between negative quality control (NQC) and average results of drug-naive human serum (NHS) from six runs. However, in Example 3, an R^2 value of 0.04 and a non-significant linear regression slope ($p = 0.69$) indicate inadequate correlation between NQC and NHS from six runs.

vidual study samples. This assumption can be verified using data from the screening-cut-point experiment by evaluating the significance of the correlation and linear relationship of the negative control readings from each of the six assay runs versus the average of the individual subject samples from the corresponding runs (Fig. 16.5). If the results are significantly correlated, this would suggest that the negative control readings vary in the same direction as the individual subject samples. If not, this negative control pool is not appropriate for defining the floating cut point during the in-study phase. Reasons for this inconsistency could be that the subjects used in producing the negative control pool are not adequately representative of the study population, or that the quality of the pooled controls may have degraded over time (e.g., if the pool originated from an old study), or other reasons. Possible solutions to this issue are to produce a new negative control that is more representative of the study population or to use a subject-specific cut point for the screening assay by using the subject's baseline sample in place of the negative control. If the latter approach is used, then it should be emphasized that any pre-existing antibodies cannot be identified. Also, while using this approach, it is important to test the baseline subject samples together with the post-treatment samples in the same assay run. Thus, the former approach, that is, to recreate a new negative control pool, is preferred. Since issues such as those just outlined that occur during the validation phase can cause considerable delays in a program, it is

recommended that the suitability of the negative control be determined during the assay development phase using the correlation analysis described in this section.

16.4.9. Additive versus Multiplicative Correction Factor

It is sometimes assumed that the multiplicative correction factor is always appropriate for defining the screening cut point when using the floating-cut-point approach (for example, when all subject sample results are divided *a priori* by negative control results, and the cut-points are evaluated on these ratios). As described in the outline of the screening-cut-point evaluation process (Section 16.4.1), the use of additive versus multiplicative correction factor for defining the screening cut point depends on whether or not it was deemed appropriate to do the data analysis using the original or log-transformed data. Use of the multiplicative correction factor is inappropriate when the distribution of the log-transformed data is not adequately normal and is more skewed than the distribution of the original untransformed data. Thus determination of the appropriateness of multiplicative versus additive correction factor should be made separately for each screening-cut-point experiment, and neither of these methods should be assumed *a priori* to be the more appropriate.

16.4.10. Suitability of the Cut Point for Other Study Populations

To determine whether the same screening cut point can be used for a different study population, distribution of the data from at least 20 subject samples from the new study population should be compared with the data from the screening-cut-point experiment obtained from the previous population. If the means and variances are not statistically significantly different, then the same screening cut point can be used. If the variances are similar, but the means are different, then the correction factor determined from the screening-cut-point experiment can be used for evaluating the floating cut point for the new study samples. However, if the variances are also significantly different, then the screening-cut-point experiment should be repeated for the new study population. This same idea can also be applied when comparing healthy subjects to relevant disease populations or for determining the suitability of the cut point when reagents change significantly over time.

16.5. CONFIRMATORY CUT POINT

Samples that are identified as positive in the screening assay should be tested in a confirmatory assay to determine whether the ADA levels are specific to the study drug. It has been common practice to spike the positive samples determined in the screening assay with an excess of the study drug; if the signal

is inhibited by over 50% relative to the unspiked sample, then the ADA sample is **confirmed** to be specific to the study drug. The 50% threshold of inhibition is subjective, however, and lacks any objective justification. Shankar et al. [5] introduced an objective approach for setting the threshold for the confirmatory assay, the so-called **confirmatory cut point**.

The confirmatory cut point is estimated from the same experiment used to evaluate the screening cut point. Samples from individual drug-naive subjects are spiked with excess drug and tested in the same plate as their unspiked counterparts. The percentage inhibition of these samples is then determined and is analyzed by following a similar flow scheme as outlined in Section 16.4.1 for evaluating the screening cut point. As most of the same considerations and methods related to evaluation of the distribution and outliers, comparison of means and variances between the assay runs, and so forth, apply also to evaluation of the confirmatory cut point, it is assumed that the reader is familiar with these details and only the particular and unique issues relating to evaluation of the confirmatory cut point are reviewed in this section.

The mean plus 2.33 × SD of the percentage inhibition is used for setting the confirmatory cut point, where 2.33 corresponds to the 1% false positive rate from a normal distribution, instead of the mean + 1.645 × SD used to evaluate the screening cut point. If a lower false positive rate such as 0.1% is adequate, then 3.09 can used in place of 2.33 in this formula.

Since negative samples spiked with excess study drug are used to calculate the confirmatory cut point, the percentage inhibition for a number of these samples may be negative. If log-transformation is necessary for the cut-point evaluation to ensure adequate normality, then taking the logarithm of a negative result is problematic. In such cases, the intermediate steps in the analyses used to evaluate the cut point should be performed on the log-transformed ratio of spiked to unspiked samples. Since the inhibition is calculated as 1 minus the ratio of spiked to unspiked samples, the 99th percentile of percentage inhibition corresponds to the 1st percentile of the ratio. Therefore, the cut point for the log-transformed ratio is defined by the mean minus 2.33 × SD. This cut point, after taking the antilogarithm, is then converted back to the percentage inhibition.

16.5.1. Fixed versus Floating Confirmatory Cut Point

Ideally, both the spiked and unspiked individual sera should be run together in the same plate to evaluate the screening and confirmatory cut points. This will often result in the ratio of spiked to unspiked samples (or percentage inhibition) not varying significantly between the assay runs due to the within-plate normalization, even if the unspiked samples themselves are significantly different. Therefore the confirmatory cut point can be a fixed cut point during the in-study phase even when the screening cut point has to be floating. Sometimes, however, the ratio of spiked to unspiked samples (or percentage inhibition) can be significantly different between the assay runs. In such cases,

a floating confirmatory cut point would be ideal during the in-study phase, instead of a fixed confirmatory cut point. An additive or multiplicative correction factor should be determined using the ratio of spiked to unspiked negative control samples; it is therefore useful to run both spiked and unspiked negative control samples in the validation experiments, and also in every plate during the screening phase if a floating-cut-point method is needed. For further details on evaluating correction factors and related topics, please refer to Section 16.4.

16.5.2. Validity of the Confirmatory-Cut-Point Method, and Alternative Approaches

Ideally, the confirmatory cut point should be evaluated using positive samples from the screening assay that are nonspecific to the study drug. It is often not feasible to obtain an adequate number of such samples. Therefore, the same individual drug-naive negative samples used in the screening-cut-point determinations are employed for this evaluation. Theoretically, this may be a reasonable approach because the inhibition of nonspecific positive samples spiked with the study drug should be quite negligible and therefore similar in distribution, in terms of both mean and variance, to the inhibition of negative samples. This can be readily verified using simple graphical techniques. The mean of the inhibition values from the six assay runs for each subject are first plotted versus the mean of their unspiked sample results. The standard deviation of the percentage-inhibition results from the six assay runs for each subject are then plotted versus the corresponding mean of the unspiked sample results (Fig. 16.6). It is then determined whether the mean and variance of the inhibition results show some linear trend across the entire range of the unspiked sample results (from low negative samples to high negative or low positive samples). This can be assessed visually and also more formally by evaluating the significance of the linear regression line of these data.

If the mean and/or variance of the inhibition results trends toward a linear response across the range of the unspiked sample results, then the confirmatory cut point should be re-evaluated using only half the unspiked samples with the highest signal along with their spiked counterparts, and compared with the cut point determined from all the samples. If the results are similar, then the confirmatory cut point can be reported using all the samples; otherwise the cut point should be reported using only this subset of samples with the highest signal.

An alternative approach for evaluation of the confirmatory cut point that has been considered by some scientists is to use at least 50 mock low positive samples instead of the negative samples. These are essentially the same samples as those used in the screening-cut-point experiment, but spiked with a low concentration of the positive control. These samples are then spiked with excess drug to determine the percentage inhibition, and then the confirmatory cut point is determined using a similar analysis as that just described. This cut

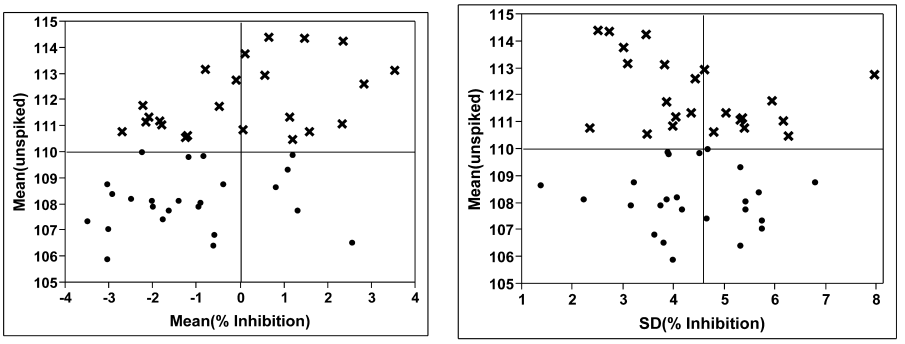


Fig. 16.6 Comparison of mean and SD of percentage inhibition across the entire range of unspiked sample results from a cut-point experiment. Percentage inhibition is different between high and low negative samples. Confirmatory cut point with all data = 8.95%; confirmatory cut point with only high negatives = 9.2%.

point is defined to be the lower 1st percentile of the distribution of the mock low positive samples. The positive samples from the screening assay obtained during the in-study phase that inhibit less than the value of this confirmatory cut point when spiked with the study drug are deemed to be nonspecific, and those that are above this cut point are considered to be confirmed positives. The following points should be carefully considered: (1) Samples used in this evaluation are not nonspecific positive samples; the low mock positive samples are highly specific to the study drug as these are negative samples spiked with the specially prepared control antibody. (2) Determination of the appropriate level of control antibody to be used to spike the negative samples in order to create the mock low positive samples is subjective; this is a critical issue as it can impact the level of inhibition and hence the value of the confirmatory cut point. (3) The control antibody used may not be similar to the true positive patient samples in terms of its affinity, avidity, and other characteristics. Thus, for these reasons, this approach seems more controversial and scientifically questionable than the negative-sample approach previously described. In addition the validity of the negative-sample approach can be readily verified and just a subset of samples can be used when problems arise.

16.6. SENSITIVITY AND LOW POSITIVE CONTROL

Assay sensitivity can be determined using, for example, five serial dilutions of a pool of a high concentration of a positive control antibody in a series of six runs using two or more analysts. The highest dilution chosen should result in a reading that falls below the screening assay cut point. The concentration of control antibody that corresponds to the screening cut point is then interpolated from each of the six individual dilution curves, and the mean and SD are

calculated. Sensitivity is often determined as the mean for 50% consistency such that reading for half of the samples will fall either above or below the cut point. It is often more useful, however, to calculate sensitivity such that there is a 95% assurance that samples will fall above the cut point, using the formula, sensitivity = mean + $t_{0.05,5df} \times$ SD, where $t_{0.05,5df}$ is the threshold corresponding to 5% error rate from the one-sided *t*-distribution with 5 degrees of freedom (6 runs minus 1). Similarly, the concentration for the lower positive control (LPC) is also determined using the same data. Again it is most useful if the LPC is calculated such that only 1% of the sample readings will fall below the cut point using the formula LPC concentration = mean + $t_{0.01,5df} \times$ SD. Alternatively, the false negative rate of a predetermined LPC concentration can be evaluated using these same data in order to justify the appropriateness of this LPC for this validation and future use.

16.7. PRECISION OF SCREENING AND CONFIRMATORY ASSAYS

To determine the precision of the screening assay, negative, low positive, and high positive control samples should be tested in every plate and in every run. By the end of the validation phase, data will be available from a total of at least 20 plates in 10 runs that can be used to determine intra- and inter-run precision or percentage coefficient of variation (%CV) for these controls. If a fixed cut point is used in the screening assay, then the precision of negative, low positive, and high positive control samples can be reported directly. If a floating cut point is used, however, then the precision of the ratio of the low positive control to negative control and the ratio of the high positive control to negative control should also be calculated.

For determining the precision of a confirmatory assay, the low positive control samples spiked with the study drug should be tested in every plate during the validation phase. The percentage inhibition of these samples, determined by comparing spiked with unspiked low positive control samples, can be used to evaluate intra- and inter-run precision.

16.8. EVALUATION OF TITER, TITRATION CUT POINT, AND PRECISION OF TITERS

To determine the titer of a confirmed positive sample, a series of dilutions of the sample are carried out. The titer of the sample is defined as the dilution factor (reciprocal of the dilution) at which the response equals the screening cut point. Although titer is often determined from the reading immediately above the cut point, this method is subject to bias and it is preferable to determine titer from the interpolation of the dilution curve. If this is not feasible, then the titer can be interpolated from the two points flanking the cut point. However, this method can be adversely affected by outliers.

If the screening cut point falls in the lower plateau of the dilution curve, then a higher cut-point value can be used to calculate the titration cut point. This higher value can be based on either 99% (2.33 × SD) or 99.9% (3.09 × SD). This titration cut point is then used to calculate titers. Confirmed positive samples that fall between the screening and titration cut point can be reported as having a titer of 1.

To determine the precision of a titer, serial two-fold dilutions of five or more mock high positive control samples should be assayed in at least six runs by two or more analysts. Mock high positive control samples can be obtained by spiking individual negative sera from the target population with a high positive control. The titer is then determined by interpolation of each of the dilution curves and the overall mean and SD are calculated. Intra-assay and interassay precision (%CV) can then be determined. Also, using these data, the minimum significant ratio (MSR), where $MSR = 10^{t \times sqrt(2) \times SD}$, can be determined, where SD is the overall standard deviation (the intra-run plus the inter-run variation) and t refers to the threshold from a two-sided Student's t-distribution with $n - 1$ degrees of freedom and 5% error rate. The calculated MSR reflects the smallest fold-change in the titer values that can be considered as statistically significant ($p < 0.05$); that is, if MSR = 5, then titers that are different by over fivefold can be considered as significant.

16.9. IN-STUDY ASSAY ACCEPTANCE CRITERIA

The data obtained from all the assay runs from the various experiments during the entire validation phase should be used to calculate the in-study assay acceptance criteria. If a fixed-cut-point approach has been adopted for the screening assay, then the in-study assay acceptance criteria can be determined from the negative, low positive, and high positive controls. This is obtained by calculating the mean plus $t_{0.005,df} \times SD$ for each negative, low positive, and high positive control, where $t_{0.005,df} \times SD$ corresponds to the threshold from a two-sided Student's t-distribution equivalent to a 0.1% failure rate. If this failure rate is too high, then it can be redefined and a different threshold from the Student's t-distribution can be used. It is important to emphasize that the standard deviation used in this formula corresponds to only the intra-run SD of the control samples.

If a floating cut point is used, then in-study assay acceptance criteria as just defined should be calculated using the ratio of the value of the low positive control samples to that of the negative samples, and the ratio of the value of the high positive control samples to that of the negative controls.

16.10. OPTIMAL "ALL-IN-ONE" PLATE LAYOUT

The proposed optimal layout illustrated in Figure 16.7 allows the maximum amount of information required for assay validation to be obtained from a

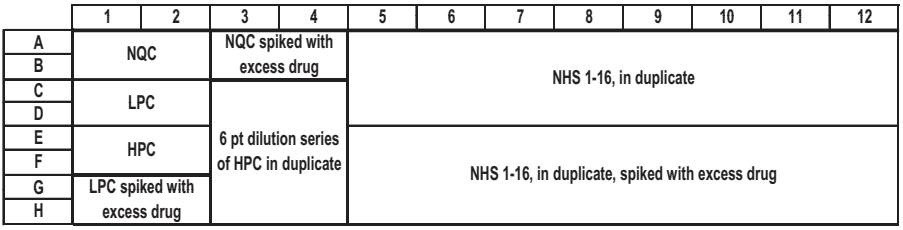


Fig. 16.7 Optimal plate layout. Using an all-in-one plate layout such as this one in the balanced-design experiment described in Table 16.1C, the following determinations can be made from a single experiment: screening cut point (CP), confirmatory CP, correction factors for screening and confirmatory CP if needed, sensitivity, concentration for LPC, precision of controls, and in-study acceptance criteria.

single plate. In the proposed layout it is recommended that 2 duplicate samples of negative control, low positive control, and high positive control, as well as 2 duplicate samples of a negative control and low positive control spiked with drug, be tested in duplicate on the plate. In addition, the plate includes a series of 6 dilutions in duplicate of a high positive control. The remainder of the plate contains 16 samples of negative sera from drug-naive individuals, in duplicate, and the same 16 samples spiked with excess drug. Thus, such a layout allows determination of screening cut point, confirmatory cut point, correction factors for the screening and confirmatory cut points, sensitivity, justification for the concentration of low positive controls, precision for screening, confirmatory, and titration assays, and in-study plate/run acceptance criteria.

For the evaluation of screening cut point this plate layout is used as part of the balanced design shown in Table 16.1C. For other validation experiments, such as sample freeze–thaw stability studies, dilution linearity studies, and robustness, a similar plate layout as just described should be used by replacing the 16 spiked and unspiked samples, necessary for the determination of screening confirmatory cut point, with other appropriate samples. Thus, on completion of the validation phase the data accumulated from the various experiments will be sufficient to determine the different types of assay precision and in-study acceptance criteria.

16.11. EVALUATION OF CUT POINT FOR NEUTRALIZING ANTIBODY ASSAYS

Recommendations on the development and validation of NAb assays have been addressed in white papers [6, 7] and book chapters [8]. Most of the statistical details related to the evaluation of cut points for NAb assays are similar to the methods described for the immunogenicity screening assays. The experimental details vary slightly and will be described here briefly.

A fixed concentration of study drug should be spiked in at least 30–50 individual negative sera, preferably from target disease population, in at least two runs and by two analysts. Evaluate the cut point from these data using the methods described in Section 16.4. During the in-study phase, the same concentration of study drug should be spiked in the patient or subject sample. This sample result is then compared to the cut point to classify the sample as either NAb positive or NAb negative.

An alternative approach that is more tedious, but potentially beneficial when there is high assay variability, is based on the evaluation of the shift in EC_{50} due to the neutralizing effect of the ADA. In this approach, varying levels of the study drug are spiked in at least 10 individual negative sera in at least two runs, by two analysts, during prestudy validation. EC_{50} is then estimated from each of these curves using a nonlinear regression model such as the four-parameter logistic model. Now a cut point of the ratio of EC_{50} values (CPR.EC_{50}) can be determined using the following formula:

$$\text{CPR.EC}_{50} = 10^{t\times\text{sqrt}(2)\times\text{SD}}$$

This is similar to the MSR evaluation for precision of titers presented in Section 16.8. Here SD is the standard deviation of the $\log_{10}$-transformed EC_{50} values, t is the threshold value from a one-sided Student's t-distribution that corresponds to 5% false positive rate and $n–r$ degrees of freedom ($t \sim 2$ for large n), and r is the number of runs. During the in-study phase, different concentrations of the study drug can be spiked in the negative control and in each of the subject samples, and the EC_{50} can be estimated for each of these samples using nonlinear regression. Calculate the ratio of EC_{50} of each subject sample versus the corresponding negative control sample from the same assay run. If this ratio is greater than the CPR.EC_{50} evaluated from the validation data, then the shift in EC_{50} of the subject sample can be considered as caused by the neutralizing effect of the ADA, and thus the subject sample is considered as NAb positive.

REFERENCES

1. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469-475.
2. Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., Kuter, D.J. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241-3248.
3. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of

immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.

4. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.S., Stebbins, C.C., and Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
5. Shankar, G., Devanarayan, V., Barrett, Y.C., Bowsher, R., Finco-Kent, D., Fiscella, M., Gorovits, B., Kirschner, S., Moxness, M., Parish, T., Quarmby, V., Shores, E., Smith, H., Smith, W., Zhong, J., Zuckerman, L., Koren, E. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–1281.
6. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
7. Gupta, S., Devanarayan, V., Finco, D., Gunn, G., Kirshner, S., Richards, S., Rup, B., Song, A., Subramanyam, M. (2011). Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics, to appear in *J Pharm Biomed Anal*.
8. Parish, T., Finco, D., Devanarayan, V. (2010). Development and validation of immunogenicity assays for preclinical and clinical studies. In *Ligand-Binding Assays*. Findlay J, Khan M, eds. Wiley, Hoboken, NJ, chap. 8.

agents act as antigens that induce neutralizing antibodies (NAbs) [1, 2]. NAbs are important clinically because their development to biopharmaceutical products can correlate with reduction in both drug bioavailability and therapeutic efficacy in patients. The measurement of NAbs is also an important part of the preclinical evaluation of possible immunogenicity of new drugs under development. Importantly for antigens with high specific activity, cell-based assays are used, since the measure of biological response can be more precise than measurement of mass. Multiple factors have been described that affect the design and optimization of cell-based assays [3] and their use in evaluating immunogenicity [4]. Desirable attributes to consider in the design of quantitative NAb bioassays include sensitivity, reproducibility, and precision, as well as the important consideration as to how assay results are expressed that allow meaningful comparisons.

The extensive experience gained over the past three decades from the study of the prototypical cytokines, the interferons, can offer helpful insights and practices that should be applicable more generally to the study and quantification of NAbs to other unrelated antigens. At least as many as a dozen different expressions of interferon neutralization results have been used, involving different methodologies; this variety of expressions and methodologies makes comparison and interpretation of the data from different laboratories difficult, if not impossible, emphasizing further the need for standardization of the measurement of NAbs. The growing acceptance in the interferon field of an approach to a common NAb unitage related to bioassay design, detailed further herein, indicates an intent to alter this situation [5, 6]. We propose that essentially the same approach to bioassay design and unitage taken for interferon NAbs be used to measure NAbs induced not only by interferons, as prototype, but also by a variety of protein effectors, such as other cytokines, growth factors, and hormones.

The theoretical analyses on the nature of the interactions between NAbs and the interferons, antigens which are cytokines with high specific activity (i.e., very potent biological activity in relation to weight), have been strongly supported by experimental findings from many laboratories [6–14]. To our knowledge, no other substantiated theoretical basis exists to explain the neutralization reaction of antibodies to such antigens. The methods and formulations derived from these analyses inform the design of neutralization bioassays and the derivation and reporting of results, allowing for a commonality of approach that can be applied to new products in development as well as to those already in clinical use.

The need for standardization has long been recognized. Standardization of the approach to bioassay design as explained herein does not restrict the choice of an investigator to a specific assay system, since the design can be applied to essentially any assay type. The standardization of the design avoids the requirement for a specific methodological protocol that stipulates a particular cell, or set of reagents, or detection technology, and so on, and further permits the application to future, newly developed assays, the results of which can be expressed in a common unitage. The standardized design does require

the internally consistent, correct measurement of antigen potency and the expression of results in the appropriate unitage.

The principles of design as applied to the two neutralization bioassay methods, the **constant antigen** (with dilution of antibody), and the **constant antibody** (with dilution of antigen), will be presented along with their utility, pertinent theoretical relationships, and the computation of results. Key terms, formatted in bold within the text, are listed for reference in the glossary at the end of this chapter. Our objectives for this chapter are to (i) describe the structural elements of the design of NAb bioassays based on theoretical considerations that have been substantiated by experimental data, (ii) provide operational guidance, and (iii) present the rationale for a standardized approach to express neutralization results. We attempt to explain in straightforward, nonmathematical language the concepts that have been previously described in depth in publications over the past three decades. Our hope is also to eliminate the misunderstandings and misapplications we have noted in past descriptions in the literature or communicated to us by colleagues that can result in inappropriately expressed NAb results.

15.2. NATURE OF THE NEUTRALIZATION REACTION

The measurement of neutralization of an induced cellular response to an antigen is necessarily indirect. It is based on reducing an observable biological effect of a known total amount of added antigen to a measurable, residual, free amount of antigen remaining after incubation with antibody. It was perhaps initially logical to assume that the neutralization reaction, observed as a concentration-dependant change in biological effect, is such that the quantity of antigen neutralized corresponds directly to the quantity of antibody. However, with the creation of potent antisera to interferons in the 1970s it was observed that the neutralization of the biological activity of interferons was not solely related to antibody concentration as mass per unit of volume.

Kawade and colleagues in the 1980s developed and tested theoretical models of the interactions between interferons and NAbs by applying the law of mass action to create a mathematical model of interferon neutralization [6–14]. These concepts and observations helped inform general recommendations about interferon neutralization utilizing the tenfold reduction approach endorsed by the World Health Organization (WHO) [15–18]. The opportunity arose in the 1990s to evaluate further the mathematical model of Kawade and colleagues from an analysis of the data obtained in a WHO international collaborative study of human serum anti-interferon-alpha and -beta antibody preparations that involved multiple laboratories and different bioassay methods [6, 7, 19, 20]. The study confirmed earlier experimental data and the validity of the theoretical model, resulting in a specific recommendation as to how titers should be calculated for interferon neutralization and reported in tenfold reduction units (TRU/mL) from appropriately designed bioassays [6, 7]. Subsequently that approach was recommended unanimously by the

Standards Committee of the International Society for Interferon and Cytokine Research for adoption by the interferon field to be used for bioassay design and reporting the results of neutralization tests in TRU/mL, especially of serum samples from patients with NAbs to interferons [5]. The formula to obtain that unitage is $t = f\,(n - 1)/9$, where t is the neutralization titer in TRU/mL, f is the antibody dilution at the endpoint, and n is the ratio of added-to-remaining antigen measured in terms of biological potency in that day's titration [6, 11, 13].

The recommendations and associated approach to bioassay design have been used extensively for the measurement of NAbs of the several, quite different, molecular types of interferons, including type I (alpha, beta), type II (gamma), and type III (lambda). The interferons share characteristics with many other cytokines, including interleukins, growth factors, and protein hormones, in possessing nonrepetitive epitopes [21, 22] and high specific activity (~10^8 biological activity units per milligram of protein) [5]. The approach to interferon NAb assay design and unitage was recently extended to the measurement of antibody neutralization of interleukin-6, a cytokine quite different from the interferons, having multiple cellular effects, including cell growth stimulation [23].

The neutralization reaction model proposed by Kawade and colleagues assumes that the binding of one molecule of antibody to one molecule of antigen results in complete inactivation of the antigen. The model describes theoretical modes of neutralization that available experimental evidence suggests are applicable to any protein effector with high specific activity and nonrepetitive epitopes. In one mode, neutralization of the antigen occurs in constant proportion to the concentration of antigen; that is, if 100 units of antigen potency is reduced to 10 units, 20 would be reduced to 2, or 10 to 1, or 5 to 0.5, and so on; such a reaction has been designated the **constant proportion (CP) mode** since, in this mode, neutralization of antigen potency occurs by proportion. Neutralization in this mode is proportionate, according to a ratio or rate, as if by division by a certain constant, and thus NAb potency is not definable in terms of antigen potency (neither in Laboratory nor in International Units). A contrasting mode of neutralization represents a fixed amount of neutralization. In that mode an antigen potency of 100 units would be reduced by antibody to 91, or 20 reduced to 11, or 10 to 1; this has been accordingly designated the **fixed amount (FA) mode** since antigen potency is neutralized, in this mode, by subtraction of a set amount. For the CP mode, which is the neutralization reaction most commonly seen with interferons, the dose–response curve slope is similar in magnitude to that of the antigen curve, whereas that of the rarely observed neutralization reactions in the FA mode have slopes at least twice as steep as that of the antigen curve when assayed with the constant antigen method [6, 7].

Neutralizing antibody potency is reported as TRU/mL for both neutralization reaction modes. If there is a homologous International Standard Preparation of antigen, the relative bioassay sensitivity to it is also to be

reported, which for FA mode neutralization reactions can be used to relate NAb potency to an appropriate International Standard [23].

15.3. CONSTANT ANTIGEN AND CONSTANT ANTIBODY METHODS

15.3.1. Elements and Considerations in Common to Both Methods

To contrast and compare the features of the two neutralization methods, the constant antigen and constant antibody methods, the characteristic **dose–response curves** of each are depicted in Figure 15.1. The neutralizations were performed on the same assay plate along with the simultaneous dose–response curve of the antigen necessary to define potency in terms of biological effect. The dose–response curves of antigen and those of antibody neutralization are sigmoidal, that is, generally linear in the central portion and curving toward the horizontal at the upper and lower limits of response. These characteristics, which relate to the underlying assay dynamic and to the units representing

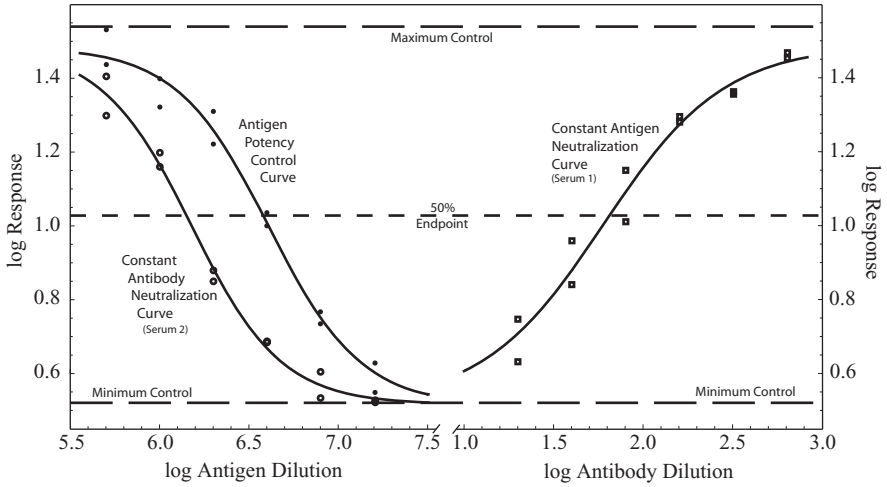


Fig. 15.1 Illustrative dose–response curves independent of a specific assay system. A constant antibody neutralization curve of one serum is shown on the left, and a constant antigen neutralization curve of another serum on the right, with the simultaneous antigen control in-between. The dose–response curves were fitted to the data by a custom-made computer application using a four-parameter nonlinear regression [8, 23]. The abscissa is in base-10 log units of the dilution of antigen or of antibody, as indicated. The ordinate, also expressed in log values, represents the measure of cellular response. The 50% endpoint is determined as half the distance between maximum and minimum values (as expressed on the ordinate) of the means of those respective controls. The calculations of antigen potency and of the NAb titers of the two sample sera are described in the text.

response and dose on the two axes, serve as the essential basis by which antigen potency is internally defined.

Cellular assays are fundamentally logarithmic in that the concentrations of both antigen and antibody vary exponentially, since dilutions are usually made in fold-dilution series, and the scale of the corresponding **cellular responses** are also exponential. The dose–response dynamic is thus best represented when the elements of both axes are expressed in logarithmic units. Note that instruments may or may not report measurements in log units: for example, absorbance or optical density (OD) units are logarithmic, whereas counts per second (CPS) or relative light units (RLU) are not and require transformation to log units. In any case, readings should not be normalized (e.g., converted to percentage), which can obscure the observed differences in magnitude.

As might be imagined from the cellular response mechanisms operative in a population of cells, there are **maximum and minimum limits** to the cellular response, as evidenced by the tendency of the ends of the dose–response curve to converge toward the horizontal. The cellular response limits serve as a basis for constructing an internal reference for defining cellular response, and thus antigen biological potency. The reference is the 50% endpoint, the midpoint of the distance between the two limits (that is, the sum of those values divided by 2). Although dose–response curves are generally constrained by these limits, in an experimental context the computed values at the asymptotes of the curve do not define the maximal and minimal limits either precisely or accurately. The **50% endpoint** is established independently of the dose–response curve in reference to the mean of replicate maximum and minimum controls (six or eight each). The maximum and minimum controls must be designed to faithfully and reproducibly represent these limits and define them through experimental determination according to the assay system.

By serving as the titration endpoint, the 50% endpoint defines one **laboratory unit (LU)** of response and thereby antigen potency in laboratory units per unit volume (LU/mL). The antigen potency is thus internally defined by the dose–response of the assay, which has the advantage of being applicable in principle to any type of biological potency assay for a given effector, such as a new biopharmaceutical under development. In the example in Figure 15.1, the antigen potency in $\log_{10}$ units of the dilution is 6.59 (rounded), the antilog of which is the **antigen titer**, 3,850,000 (rounded), such that in this titration the **antigen stock** (i.e., the undiluted antigen) has an apparent potency of 3,850,000 LU/mL, and from this the potency at any dilution can be calculated.

The precise, simultaneous determination of the amount of biologically active antigen in a particular assay titration in terms of biological effect is especially important for antigens of high specific activity, since a small change in the physical amount of antigen results in a large change in biological response. For example, for the interferons there are ~10^8 biological activity units per milligram of purified protein or ~10^{-12} M at 1 LU/mL; it is important to note that such values are an approximation and cannot be used to define

accurately the potency of antigen for immunological tests [5]. Likewise, although it may seem reasonable, perhaps even desirable, when International Standard Preparations are available to design a bioassay such that antigen potency is defined in terms of **International Units (IU)** of biological activity, it must be remembered that the IU of an International Standard is a value assigned by consensus of a WHO committee and based on data from multiple laboratories. Thus, unlike an LU, an IU value is not a measure of actual antigenic potency in a given titration and does not meet the criterion of an internal measure of antigenic biological activity.

The **relative sensitivity** of a given bioassay to an International Standard Preparation of antigen is similarly a value determined as the mean of repeated measurements (at least five) of the IU/LU ratio of the assay for that International Standard. This determination should be reported as part of the description of bioassay methodology, which allows the conversion of LU to IU of a protein effector product [7].

Since for the neutralization assay the quantities of biologically active antigen molecules involved are very small within the linear portion of the dose–response curve, the 50% endpoint is used as the precise common point of reference in laboratory units of response, that is, antigen potency, applied directly to the neutralization dose–response curves run on the same plate as the antigen potency curve. The endpoint for the neutralization reaction is 1 LU/mL, not zero, which is not measurable; thus the antigen potency is reduced to 1 LU/mL as a measure of remaining free antigen.

15.3.2. Constant Antigen (kAg) Method

The constant antigen method, in which antibody is varied, is the most commonly used, time-honored approach taken in most microbiological antibody assays: the sample with antibodies is diluted out beyond an expected endpoint and a targeted amount of antigen is added to each diluted sample. Indeed, for measuring antibodies to the various interferons, the kAg method has been the approach most commonly used for decades in laboratories worldwide. Characteristic of this methodology is a neutralization dose–response curve that is opposite in slope to that of the antigen curve (Fig. 15.1), since as the antibody is diluted out, the amount of free antigen increases and thus also the observable biological response. The slope and quality of the neutralization curve are also affected by the concentration of antigen added to the sample containing antibodies: too much antigen reduces the sensitivity to detect NAbs, and too little antigen reduces the reliability of the assay. For example, interferon doses of 4 LU/mL or less produce unreliable NAb measurements [8]. Clearly an objective is to use a quantity of antigen that results in a neutralization curve that adequately covers much of the dynamic range between the minimum and maximum responses.

To measure neutralization, an experimentally determined amount of antigen, considered to be the total amount of antigen, is added to the sample

with antibody. In the example illustrated in Figure 15.1, the final target concentration of antigen is 10 LU/mL. Based on measurements taken earlier, that potency was estimated to be near the dilution at log 5.7, the antilog of which is 501,187 (rounded). The dilution of added antigen that was selected was thus 1:500,000. Given that the stock antigen potency actually measured is 3,850,000 LU/mL, as previously mentioned, the actual potency in LU/mL of total antigen is 1:500,000 of 3,850,000 LU/mL, or 7.7 LU/mL in this illustration.

Since the titration endpoint of the neutralization curve is also the 50% endpoint, by definition there is 1 LU/mL of un-neutralized, free antigen at that antibody dilution. In the case of Serum 1 in Figure 15.1, that dilution of antibody in log units is 1.81 (rounded), the antilog of which is 65.2 (rounded).

The slope of the antigen curve depicted in Figure 15.1 is –0.913, near the ideal slope magnitude of 1 to allow for meaningful comparison to the NAb curve. The constant antigen neutralization curve slope is 0.706, indicating that the neutralization reaction is in constant proportion since the magnitude of both slopes is similar. In these cases, slope is calculated by computing the change in cellular response over the length of a serial dilution interval of the antigen centered on the 50% endpoint. [This calculation is in contrast to determining the slope at a single point, such as at the curve's inflection point (i.e., the so-called Hill slope) or elsewhere on the curve, by using the first derivative of the function that the nonlinear regression had found that best fit the data.]

From the measurement of (i) the total amount of antigen in LU/mL and (ii) the dilution of the antibody sample resulting in a remaining antigen potency of 1 LU/mL, the neutralization titer is calculated in tenfold reduction units (**TRU**)/mL according to the formula $t = f\,(n - 1)/9$, that is, *Ab_Dilution* (*Ag_Total* – 1)/9, where *Ab_Dilution* is (*f*) the dilution of antibody at the endpoint, and *Ag_Total* is (*n*) the amount of antigen, measured in LU, mixed with antibody. For the constant antigen neutralization curve shown in Figure 15.1, the NAb potency for Serum 1 would be calculated as 65.2(7.7 – 1)/9, which yields 48.5 TRU/mL.

Table 15.1 lists the ranges of potentially measurable NAb titers, calculated (without rounding) from the formula $t = f\,(n - 1)/9$, of Constructs A through F. The constructs are selections of different antigen doses and antibody dilutions. As can be seen in Constructs A–D, the range of titers that can be measured in a six-dilution titration using the constant antigen method at 10 LU/mL is numerically the same as the range of dilutions of the sample with antibody. The use of antigen concentrations below 10 LU/mL is not recommended since the dynamic range of the response curve may be compromised at such antigen levels, and thereby the reliability of the measurements [8]. However, it is possible to use dilutions less than 1:20 of antibody, as in Construct D, for the measurement of monoclonal antibody cell culture supernatant fluids or as purified immunoglobulin but it is not recommended for use with sera, which can be toxic to cells at dilutions less than 1:20 [8]. Construct A includes the

TABLE 15.1 Illustrative Constant Antigen Method Bioassay Constructs

Antigen Concentrations, Antibody Dilutions, and Potentially Measurable Titers from the Relationship $t = f\,(n - 1)/9$

Construct	Antigen (LU/mL)	Antibody Dilutions (6-step)	Titer Range[a] (TRU/mL)
A	10	20, 40, 80, 160, 320, 640	20–640
B	10	320, 640, 1280, 2560, 5120, 10240	320–10240
C	10	5120, 10240, . . . , 81920, 163840	5120–163840
D[b]	10	2[b], 4[b], 8[b], 16[b], 32, 64	2–64
E[c]	20[c]	20, 40, 80, 160, 320, 640	42–1351
F[c]	100[c]	20, 40, 80, 160, 320, 640	220–7040

Shaded areas indicate constructs generally not recommended.

[a]To illustrate the calculation, titers are not rounded.

[b]Not for use with sera which at low dilutions may be toxic for cells; potentially useful for monoclonal antibody preparations.

[c]Increasing the antigen dose is generally less practical for measuring higher titers than increasing the dilution of the sample.

lowest range of serum titers that can be reliably measured with the kAg method. Constructs B and C are useful for higher titers, which is achieved more simply, reliably, and efficiently by increasing the dilution of the antibody sample (compare Constructs A, B, and C) than by increasing the concentration of antigen (compare Construct B to Construct E, and Construct C to Construct F). Serum titers with interferon NAb titers of over 100,000 TRU/mL have been successfully measured using a target concentration of interferon of 10 LU/mL and appropriately high dilutions of serum [24].

15.3.3. Constant Antibody (kAb) Method

The constant antibody, varying antigen, method, in contrast to the constant antigen method, is very reliably sensitive; it can detect titers as low as 1–2 TRU/mL using a 1:20 dilution of serum [8]. The technique involves adding a fixed dilution of the antibody sample to a dilutional series of the antigen. Characteristic of this method is a NAb dose–response curve that parallels that of the antigen curve (Fig. 15.1), since it is the antigen that is being serially diluted. The great sensitivity of the kAb method, which is more than 10-fold greater than that of the kAg method as predicted by theory and substantiated experimentally [8], recommends its use in testing low-potency monoclonal antibodies as well as in the detection of NAbs in patient sera, and thus it is ideal to utilize for screening purposes.

In the case of patients being treated with interferon-beta products, approximately two-thirds of sera submitted for interferon-beta NAb testing had been considered negative by the kAg method at a cutoff less than 20 TRU/mL. The

testing by the kAb method of sera, previously considered to be negative, from 34 patients treated with Rebif or Betaseron showed that ~50% had detectable NAbs, whereas 10 sera from Avonex-treated patients had titers of less than 1 TRU/mL [8]. It should be noted that a titer of 20 TRU/mL indicates a reduction of activity by 200-fold, whereas a titer of 1 TRU/mL indicates a 10-fold reduction.

In the constant antibody neutralization curve depicted in Figure 15.1, a dilution of 1:20 of the antibody sample (Serum 2) was used. From the intersection of the antigen curve with the endpoint, the **antigen stock** has an apparent potency of 3,850,000 LU/mL, as previously mentioned. The intersection of the kAb neutralization curve with the 50% endpoint is near 6.15 $\log_{10}$ (in contrast to that of the antigen curve where it is near 6.59 $\log_{10}$). Thus, the potency of antigen reduced to 1 LU/mL is about 1:1,410,000 of 3,850,000 LU/mL, or 2.73 LU/mL (rounded). The neutralization titer is calculated in tenfold reduction units (**TRU**)/mL according to the formula $t = f\,(n - 1)/9$, or as $Ab\_Dilution\,(Ag\_Dilution - 1)/9$, where *Ab_Dilution* is (*f*), the dilution of the antibody sample, and *Ag_Dilution* is (*n*), the antigen potency in LU/mL of the dilution at which the neutralization curve intersects the 50% endpoint. Thus, for the kAb neutralization shown in Figure 15.1, the neutralization titer for Serum 2 would be calculated as $20(2.73 - 1)/9$ and quantified as 3.8 TRU/mL (using the rounded values given).

A sampling of the ranges of NAb titers that can be measured in a six-dilution titration using the constant antibody method is presented in Table 15.2, calculated (without rounding) from the formula $t = f\,(n - 1)/9$. Constructs A and B use antigen concentrations that are six times greater than those used in the simultaneous antigen control curve. At these greater antigen concentra-

TABLE 15.2 Illustrative Constant Antibody Method Bioassay Constructs

Antibody Dilution, Antigen Concentrations, and Potentially Measurable Titers from the Relationship $t = f\,(n - 1)/9$

Construct	Antibody Dilution	Antigen Concentrations in LU/mL	Titer Range[a] (TRU/mL)
A	20	64, 32, 16, 8, 4, 2	2.2–140
B	1200	64, 32, 16, 8, 4, 2	133–8400
C	72000	64, 32, 16, 8, 4, 2	8000–504000
D[b]	1200	1024[b], 512[b], 256, 128, 64, 32	4133–136400
E[c,d]	2[c]	10, 5, 2.5, 1.25, 0.625, 0.3125	~0.22[d]–2
F[d]	20	10, 5, 2.5, 1.25, 0.625, 0.3125	~2.2[d]–20

Shaded areas indicate constructs generally not recommended.

[a]To illustrate the calculation, titers are not rounded.

[b]Not recommended if very high antigen concentrations are toxic.

[c]Not for use with sera which at low dilutions may be toxic for cells; potentially useful for monoclonal antibody preparations.

[d]Generally not recommended due to the difficulty in practice of discerning at the lower limit a difference between the curves for the antigen control and antigen–antibody mixture.

tions a larger range of NAb titers can be measured than can be done at antigen concentrations starting at 10 LU/mL, such as those of Constructs E and F which can measure comparatively limited NAb ranges, but of very low titer. Constructs A and F, having a sample dilution of 1:20, provide the greatest possible sensitivity for testing sera. The lowest detection limit for Constructs E and F is constrained by the requirement for a reduction to 1 LU/mL and the ability in practice to discern a difference between the curves for the antigen control and antigen–antibody mixture. Constructs C and D accommodate measuring higher titers than those covered by Constructs A and B, but Construct D might be impractical for testing samples with unknown quantities of antibody if the very high concentrations of antigen were toxic.

15.4. ELEMENTS OF DESIGN

The use of the tenfold reduction unitage approach to bioassay design with its calculation of NAb potency should have application to the neutralization of any cytokine, growth factor, protein hormone, or similar effector molecule of high specific activity for which a cellular effect can be quantified with adequate sensitivity. Fundamental to the methodology is relating neutralization directly to an observable, objective, biological effect of the antigen rather than to arbitrarily selected weights of antigen, to the extent that selection by mass can ever be made precisely at the low levels required with antigen of high specific activity. Also of technical importance is the construction of the 50% endpoint relative to the mean of several (six or eight), replicate maximum and minimum controls, rather than the few data points from which the upper and lower asymptotes of the fitted dose–response curve are extrapolated. From the understanding of neutralization reactions, it is clear that determination of the target amount of antigen to be added is also a necessary investigative step in the assay design process.

Particular antigens and assay systems may necessitate differing implementation of the necessary assay elements. For example, the maximum and minimum controls of antiviral assays are based on a different dynamic from those of a gene-reporter luciferase assay. In interferon antiviral assays the maximum response is taken as the absorbance of dye in healthy cell populations, untreated with interferon and unchallenged by virus, and the minimum response that in virus-infected cell populations unprotected by interferon. In contrast, in interferon luciferase assays the proper maximum control is the induced light responses of cell populations treated with a maximally tolerated interferon dose, perhaps in the range of 50–200 LU/mL, and the minimum control that of cell populations not exposed to added interferon. Another possible assay-dependent design consideration is the amount of target antigen to add, which for interferons the WHO recommendation is 10 LU/mL [15–18], the same target dose that was used with a very different antigen, interleukin-6 (IL-6) [23].

15.5. DISCUSSION

The standardization approach to bioassay design described herein for the measurement of NAbs to biopharmaceutical effectors of high specific activity allows for a commonality in the expression of results as TRU/mL. It avoids the problem of defining NAb unitage in terms of the component nature of an assay system, its specific technique, particular cells, reagent sets, methods of detection, etc. Instead, the approach requires that the design of the assay allow for the internally consistent, simultaneous measurement of biological effect as antigen potency, permitting the expression of results in unitage defined by a standardized bioassay design. This general approach has been used for decades to measure NAbs to interferons of quite different biological and genetic nature as well as NAbs to a very different cytokine, IL-6, recently demonstrated as proof of principle for application to different protein effectors [23]. The general approach has been repeatedly endorsed for interferon NAbs in the 1980s and 1990s by different WHO committees [15–18].

Although the WHO recommendations were lacking in methodological specifics, which this chapter and our previous publications attempt to remedy, interferon laboratories worldwide proceeded eventually to adopt the general tenfold reduction approach to measuring interferon NAbs. In 2001 we reported the analysis of an international collaborative assay study, in which multiple laboratories using different bioassays to measure NAbs to Type I interferon-alpha and interferon-beta, that confirmed the theory and earlier experimental findings of Kawade and colleagues [6, 7]. Such methodology was equally applicable to a very different molecule, interferon-gamma [25]. In 2003 an international group of neurologists meeting in London under the auspices of the Consortium of Multiple Sclerosis Centers strongly endorsed the TRU approach to measure IFN-beta NAbs [26]. The European Medicines Agency (EMEA) also endorsed this general approach [27, 28] but details were not provided. We have tried in this chapter to clarify elements in design not necessarily apparent in our previous publications and have suggested herein some improvements and refinements in bioassay methodology applicable to interferons and other protein effector molecules.

From the earliest days of immunology, neutralization tests of toxins of bacteria, plants, insects, or reptiles were measured and defined by the specific tests for the biological activities of the antigens measured, in which the animal or cell, antigen dose used, or the nature of the biological effect to be measured were necessarily required to be specified. The discovery of soluble cellular or tissue factors (such as the interferons) with very high specific activities attributable in large part to the triggering, after receptor binding, of multiplier, molecular cell networks further complicated the detection and measurement of NAbs. The multiplicity of effects of protein effectors, entities such as interferons, interleukins, or growth factors, have made it possible to create assays definable by such effects, which in turn open vistas of expanding complexity to express the results in many different ways. The approach we describe for

standardizing NAb assay design and reporting of results in TRU allows for the application of current and future assay techniques, instrumentation, and materials such that the results are interpretable and comparable.

It is now generally appreciated that the development of neutralizing antibodies to pharmacologically active biological products can reduce drug availability and impair therapeutic efficacy in patients. For example, about a third of patients receiving parenteral interferon injections several times a week for years develop NAbs against the interferon product administered, with the likely loss of drug bioavailability [29] and clinical efficacy [30, 31]. Apparent titers may fluctuate and clinical decisions may be determined by such information. Over the decades since the discovery of the interferons and other similar protein effectors of high specific biologic activity, many bioassays of very different types, designs, and variations in the expressions of NAb results were created to detect and quantify these antibodies, making it difficult or impossible to interpret and compare the results of different laboratories. In earlier years, there had been a tendency of scientists (at least in the interferon field) who created new assays, like the great chefs, to ardently proclaim the superiority of their own cuisine or the sale of their own recipes. There are now extant a number of excellent, technically acceptable, bioassay methods, and there may very well be even better ones created in the future.

The use of terms such as "neutralizing units" (NU), assigned to results obtained by very different techniques, methodologies, and endpoints continues to be a source of confusion [5]; the usage of such variably defined terms as NU has led on occasion to the pooling of quite heterogeneous data as if they were comparable. These somewhat chaotic situations have sometimes posed problems for international governmental bodies, regulatory agencies, scientific committees, pharmaceutical companies, as well as individual investigators, with the potential for scientific, economic, and legal consequences. It is hoped that the approach described in this chapter can remedy such a situation with a common method of expressing results based on a structurally common design method to make results more readily comparable, no matter the specific assay system employed.

Comparability of results by the use of the tenfold reduction approach has largely been achieved with the interferons of different biological types: type I alpha and beta interferons; type II, or gamma, interferon; and the distinctive type III, or lambda, interferons. The tenfold reduction unit has been successfully applied to very different methodologies to measure anti-interferon NAbs involving different biological effects, for example, antiviral [32], induction of MxA protein [33, 34] or of its mRNA [35], cell growth inhibition [21], or luciferase reporter gene induction [36, 37]. Indeed, NAb titers calculated as TRU/mL determined on the same serum samples by luciferase gene induction and by antiviral assay in different laboratories were quite comparable [31]. As further proof of principle, the same approach has been taken to measure NAbs to IL-6 as growth factor [23] and to tumor necrosis factor (M. Tovey, personal communication).

15.6. SUMMARY AND RECOMMENDATION

Over the past three decades analyses of the nature of the interactions between neutralizing antibodies (NAbs) and antigens of high specific activity, typified by the interferons and interleukins, have provided a firm theoretical and experimental foundation for the use of the tenfold reduction unit (TRU) as a measure of NAbs to antigens such as cytokines, growth factors, protein hormones, and other protein effectors. The TRU measure of NAb potency can be used to express the results of both types of neutralization bioassay methodology, constant antigen and constant antibody, and is otherwise assay-system-independent, provided that the assay is of sufficient sensitivity and the elements of the assay are appropriately designed, controlled, and calculated, as described, to allow expression of results in TRU/mL. If this approach is taken, it may be helpful for the ensuing few years if investigators calculate and report neutralizing antibody titers not only in TRU/mL but also as they may have done previously, for the sake of continuity with previously published data and their possible reassessment.

GLOSSARY

Antigen Stock: The preparation of antigen with biological activity from which dilutions of antigen are made for use in antigen and NAb assays. The concentration in LU/mL of any given dilution of stock is the antigen titer divided by the given dilution X (where X is the variable in 1:X describing the dilution).

Antigen Titer: The dilution of antigen for which the cellular response is halfway between maximum and minimum (i.e., the 50% endpoint, 1 LU); thus for the antigen control, the observed potency in LU/mL of the antigen stock used for a titration.

Cellular Response: The unit of measure of the instrument used to read *in vitro* biological activity [e.g., optical density (OD) / absorbance, relative light units (RLU), counts per second (CPS)], expressed in log units but not normalized, e.g., converted to a percentage. Depending on the instrument, readings may already be expressed as a log value (e.g., OD).

Constant Antibody (kAb) Method: The neutralization bioassay method in which the serum dilution or antibody concentration is held constant and a series of concentrations of antigen that is simultaneously measured is added to determine the neutralization titer of the sample.

Constant Antigen (kAg) Method: The neutralization bioassay method in which a concentration of antigen that is simultaneously measured is added

to a series of dilutions of serum or antibody to determine the neutralization titer of the sample.

Constant Proportion (CP) Mode: An antigen-antibody interaction resulting in neutralization in which antibody reduces antigen biological activity in a set ratio or constant proportion of added-to-residual biologically active antigen (e.g., 100 to 10, 20 to 2, etc.). The slope of the neutralization curve in CP mode is similar to that of the antigen dose-response curve.

Dose–Response Curve: The curve fitted to the assay data points representing log dilution (as the controlled variable) and the corresponding observed cellular response, also as a log value. The function fitted to the curve using non-linear regression is used to determine titers.

50% Endpoint: The observed cellular response halfway between maximum and minimum; the reference point which defines one LU of antigen potency.

Fixed Amount (FA) Mode: An antigen-antibody interaction resulting in neutralization in which antibody acts to neutralize a fixed amount of biologically active antigen (e.g., 100 to 91, 20 to 11, etc.). The slope of the neutralization curve in FA mode is much greater (>2×) than that of the antigen dose-response curve.

International Unit (IU): The biological activity value based on an international collaborative assay study and assigned by a WHO Committee to an International Standard Preparation of antigen, as IU/ampoule, to be reconstituted usually in one milliliter.

Laboratory Unit (LU): The antigen potency value determined in each assay titration, by which 1 LU/mL is defined as the dilution for which the cellular response is halfway between maximum and minimum limits (i.e., the 50% endpoint).

Maximum and Minimum Limits: These upper and lower limits of biological response are determined by taking the mean of measurements of multiple, appropriately designed controls.

Relative Sensitivity: A ratio expressing the sensitivity of a bioassay for an antigen product (e.g., an interferon), established by comparing (in ≥5 titrations) the observed potency, expressed in LU/mL as measured in that particular assay, of the homologous WHO International Standard, relative to its assigned potency in IU, and stated as a fraction or multiple. Not to be confused with the sensitivity of a NAb bioassay for its ability to detect NAbs, usually expressed in qualitative terms (e.g., high or low).

TRU: Tenfold reduction units of neutralization expressed per mL, as determined by: $t = f\ (n - 1)\ /\ 9$ where t = NAb titer as TRU/mL, f = antibody dilution, and n = the ratio of added antigen potency to remaining antigen potency (thus n simplifies to the measured number in LU/mL of added antigen when the reduction is to 1 LU/mL).

REFERENCES

1. Porter, S. (2001). Human immune response to recombinant human proteins. *J Pharm Sci*, *9*, 1–11.
2. Schellekens, H. (2003). The immunogenicity of biopharmaceuticals. *Neurology*, *61*, 11–12.
3. Meager, A. (2006). Measurement of interferon activities. In *The Interferons: Characterization and Application*, Meager A, ed. Wiley-VCH, Weinheim, chapter 12, pp. 339–373.
4. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., et al. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
5. Grossberg, S.E., Kawade, Y. (2006). The development and measurement of antibodies to interferon. In *The Interferons: Characterization and Application*. Meager A, ed. Wiley-VCH, Weinheim, chapter 13, pp. 375–399.
6. Grossberg, S.E., Kawade, Y., Kohase, M., Yokoyama, H., Finter, N. (2001). The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. *J Interferon Cytokine Res*, *21*, 729–742.
7. Grossberg, S.E., Kawade, Y., Kohase, M., Klein, J.P. (2001). The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. *J Interferon Cytokine Res*, *21*, 743–755.
8. Grossberg, S.E., Kawade, Y., Grossberg, L.D. (2009). The neutralization of interferons by antibody. III. The constant antibody bioassay, a highly sensitive quantitative detector of low antibody levels. *J Interferon Cytokine Res*, *29*, 93–104.
9. Grossberg, S.E., Kawade, Y. (1997). The expression of potency of neutralizing antibodies for interferons and other cytokines. *Biotherapy*, *10*, 93–98.
10. Kawade, Y. (1980). An analysis of neutralization reaction of interferon by antibody: a proposal on the expression of neutralization titer. *J Interferon Res*, *1*, 61–70.
11. Kawade, Y., Watanabe, Y. (1984). Neutralization of interferon by antibody: appraisals of methods of determining and expressing the neutralization titer. *J Interferon Res*, *4*, 571–584.
12. Kawade, Y., Watanabe, Y. (1985). The nature of neutralization reaction between effector protein and monoclonal antibody: a quantitative study of neutralization characteristics of anti-interferon antibodies. *Immunology*, *56*, 489–495.
13. Kawade, Y. (1986). Quantitation of neutralization of interferon by antibody. *Methods Enzymol*, *119*, 558–573.
14. Kawade, Y., Finter, N., Grossberg, S.E. (2003). Neutralization of the biological activity of cytokines and other protein effectors by antibody: theoretical formulation of antibody titration curves in relation to antibody affinity. *J Immunol Methods*, *278*, 127–144.
15. Andzhaparidze, O.G., Ayres, J.J., De Maeyer, E., Finter, N., Friedman, R., Grossberg, S.E., Kramer, S.M., Laughlin, C.A., Leibowitz, P.J. (1988). WHO Expert Committee

on Biological Standardization, thirty-eighth report, annex l. Standardization of interferons: report of a WHO informal consultation. *World Health Organ Tech Rep*, *771*, 37–87.

16. Berg, K., Billiau, A., De Maeyer, E., Finter, N., Galasso, G.J., Grossberg, S.E., Hilfenhaus, J., Kawade, Y., de J. Limonta, M., Meager, A., et al. (1983). WHO Expert Committee on Biological Standardization, thirty-third report, annex 1. Standardization of interferons. *World Health Organ Tech Rep*, *687*, 35–60.
17. Billiau, A., Dennis, P., De Maeyer, E., Finter, N., Galasso, G.J., Grossberg, S.E., Hilfenhaus, J., de J. Limonta, M., Meager, A., Oden, E.M., et al. (1985). WHO Expert Committee on Biological Standardization, thirty-fifth report, annex 1. Standardization of interferons. *World Health Organ Tech Rep*, *725*, 28–64.
18. Calam, D.H., Drozdov, S.G., Gust, I.D., Hardegree, M.C., Lemoine, P.E., Lyng, J., Ofosu, F.A., Tan, I., Zhou, H.-J.. (1995). WHO Expert Committee on Biological Standardization, forty-fifth report, annex 4. Biological substances: international standards and reference reagents. *World Health Organ Tech Rep*, *858*, 93.
19. Grossberg, S.E., Jameson, P., Taylor, J.L., Greiff, D. (1994). Freeze-dried Human Anti-Human Interferon-Beta Antibody reference catalog number G037-501-572. NIH Research Reference Reagent Note No. 44.
20. Grossberg, S.E., Jameson, P., Taylor, J.L., Greiff. D. (1994). Freeze-dried Human Anti-Human Interferon-Beta Antibody reference catalog number G038-501-572. NIH Research Reference Reagent Note No. 45.
21. Redlich, P.N., Grossberg, S.E. (1989). Analysis of antigenic domains on natural and recombinant human IFN-beta by the inhibition of biologic activities with monoclonal antibodies. *J Immunol*, *143*, 1887–1893.
22. Runkel, L., deDios, C., Karpusas, M., Baker, D., Li, Z., Zafari, M., Betzenhauser, M., Muldowney, C., Miller, S., Redlich, P.N., Grossberg, S.E., Whitty, A., Hochman, P.S. (2001). Mapping of interferon-β epitopes important for receptor binding and activation: comparison of results achieved using antibody-based methods and alanine substitution mutagenesis. *J Interferon Cytokine Res*, *21*, 931–941.
23. Grossberg, S.E., Casey, M., Grossberg, L.D. (2009). Quantification of the neutralization of cytokine biological activity by antibody: the ten-fold reduction bioassay of interleukin-6 as growth factor. *J Interferon Cytokine Res*, *29*, 421–426.
24. Grossberg, S.E., Oger, J., Grossberg, L.D., Gehshan, A., Klein, J.P. (2011). Frequency and magnitude of interferon β neutralizing antibodies in the evaluation of interferon β immunogenicity in multiple sclerosis patients. *J Interferon Cytokine Res*, *31*, 337–344.
25. Grossberg, S.E., Jameson, P., Taylor, J.L., Greiff, D. (1995). Freeze-dried Recombinant Human Interferon Gamma reference catalog number Gxg01-902-535. NIH Research Reference Reagent Note No. 43.
26. Pachner, A.R. (2003). Anti-IFN-beta antibodies in IFN-beta-treated MS patients. *Neurology*, *61*, S1-S5.
27. Sorensen, P.S., Deisenhammer, F., Duda, P., Hohlfeld, R., Myhr, K.M., Palace, J., Polman, C., Pozzilli, C., Ross, C. (2006). Use of anti-interferon beta antibody measurements in multiple sclerosis. In *European Handbook of Neurological Management*. Hughes R, Brainin M, Gilhus NE, eds. Wiley-Blackwell, chapter 8, pp. 72–86.

28. Trouvin, J.H., Biologics Working Party (2008). Beta-interferons and neutralising antibodies. EMEA/CHMP/BWP/580136/2007. Available at http://www.emea.europa.eu/pdfs/human/bwp/58013607en.pdf
29. Hesse, D., Sellebjerg, F., Sorensen, P.S. (2009). Absence of MxA induction by interferon β in patients with MS reflects complete loss of bioactivity. *Neurology*, *73*, 372–377.
30. Hartung, H.P., Polman, C., Bertolotto, A., Deisenhammer, F., Giovannoni, G., Havrdova, E., Hemmer, B., Hillert, J., Kappos, L., Kieseier, B., et al. (2007). Neutralising antibodies to interferon beta in multiple sclerosis: expert panel report. *J Neurol*, *254*, 827–837.
31. Cohen, B.A., Oger, J., Gagnon, A., Giovannoni, F. (2008). The implications of immunogenicity for protein-based multiple sclerosis therapies. *J Neurol Sci*, *275*, 7–17.
32. Grossberg, S.E., Taylor, J.L., Siebenlist, R.E., Jameson, P. (1986). Biological and immunological assays of human interferons. In *Manual of Clinical Immunology*. Rose NR, Friedman H, Fahey JL, eds. ASM Publishing, pp. 295–299.
33. Pungor, E. Jr., Files, J.G., Gabe, J.D., Do, L.T., Foley, W.P., Gray, J.L., Nelson, J.W., Nestaas, E., Taylor, J.L., Grossberg, S.E. (1998). A novel bioassay for the determination of neutralizing antibodies to IFN-beta1b. *J Interferon Cytokine Res*, *18*, 1025–1030.
34. Files, J.G., Hargrove, D., Delute, L., Cantillon, M. (2007). Measured neutralizing titers of IFN-beta neutralizing antibodies (NAbs) can depend on the preparations of IFN-beta used in the assay. *J Interferon Cytokine Res*, *27*, 637–642.
35. Bertolotto, A., Sala, A., Caldano, M., Capobianco, M., Malucchi, S., Marnetto, F., Gilli, F. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321*, 19–31.
36. Lallemand, C., Mariete, J., Erickson, R., Grossberg, S.E., Roullet, E., Lyon-Caen, O., Lebon, P., Tovey, M.G. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
37. Lam, R., Farrell, R., Aziz, T., Gibbs, E., Giovannoni, G., Grossberg, S., Oger, J. (2008). Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFNbeta in multiple sclerosis patients. *J Immunol Methods*, *336*, 113–118.

Immunogenicity testing uses a tiered testing approach. Initially, samples are analyzed using an immunoassay measuring neutralizing and non-neutralizing antibodies. Samples testing positive in the screening immunoassay may then be further characterized for abundance (e.g., titer, units per milliliter) and neutralizing capacity. In general, the extent of characterization of the anti-drug immune response is determined case-by-case, based on the potential impact of immunogenicity. There are several white papers detailing strategies for development of an immunogenicity testing strategy based on product risk factors [5–7].

Neutralizing antibodies block biological activity by directly binding within or near the active site, inhibiting the drug from binding its target. In addition, NAbs can bind to an unrelated site and hinder activity by inducing a conformational change. As described previously, NAbs may result in clinical consequences or require increased drug concentrations to realize the same clinical benefit. Therefore, for programs where NAbs pose a risk to patient safety or drug efficacy, an assay to assess the ADA neutralizing capacity of patient samples is critical to the drug development plan. The purpose of this type of assay is to determine whether ADAs detected by a screening immunoassay exert inhibitory effects on a drug's biological activity i*n vitro* and to evaluate any correlation to clinical observations. Therefore, in order to understand the potential implications of NAbs on drug safety and efficacy, it is important to develop a reliable assay that can detect NAbs with appropriate sensitivity and specificity. This chapter reviews current industry guidance and regulations, reviews parameters to consider when developing and validating a cell-based NAb assay, and discusses standardization of assays.

14.2. IMMUNOGENICITY TESTING STRATEGY

Shankar et al. [6] proposed an immunogenicity bioanalytical testing strategy for preclinical and clinical studies based on the level of risk associated with the product and the potential risk of ADA-related sequelae. However, Schneider et al. [8] warn that this type of approach for immunogenicity evaluation may not be appropriate in all cases and may not provide sufficient data to support the benefit–risk analysis required for licensing. Therefore, while the testing strategy proposed by Shankar et al. [6] may provide a starting point for issues to consider when developing the immunogenicity testing strategy, each drug will require individual evaluation to determine the optimal testing strategy to meet regulatory requirements. The proposed risk-assessment strategies affect the ADA testing scheme in terms of when to incorporate a neutralizing-antibody assessment. Following is a brief review of when it may be relevant to incorporate a NAb assay(s) into the immunogenicity strategy for preclinical and clinical studies supporting protein therapeutics. The consideration for using a NAb assay for studies should be evaluated independently for each program to determine the appropriate level of testing, and input from regulatory authorities is advised.

14.2.1. Preclinical

Per the Addendum to ICH S6: Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) [9], current measurement of ADAs in nonclinical studies is not routinely warranted if there is evidence of sustained pharmacodynamic activity, no unexpected changes in the pharmaco/toxicokinetics of the test article during the dosing or recovery phase, and/or no evidence of immune-mediated reactions (immune complex-related, vasculitis, anaphylaxis, etc.). However, samples should be collected during studies in the event that analysis is warranted. When study results suggest there is a need to understand immunogenicity to interpret study data, potential for immunogenicity antibody detection assays should be conducted to evaluate the presence of ADAs. When ADAs are detected, the effect on the study results should be assessed, including effects on PK and drug clearance, pharmacology effects, and toxicity. Characterization, specifically of neutralizing potential, is generally not warranted, particularly if adequate exposure and pharmacological effect can be demonstrated by a pharmacodynamic marker of activity in the *in vivo* toxicology studies. In the event that neutralizing antibody assessment is deemed appropriate to interpret the study findings, assessment of neutralizing activity can be addressed indirectly with *ex vivo* bioactivity assay, a combination of assay formats for PK-PD, or directly in a specific neutralizing antibody assay. In most cases, a screening assay followed by endpoint titer analysis may be sufficient. For certain types of protein therapeutics, NAb data may be helpful in terms of evaluating the potential consequences of NAb formation as well as providing information on drug activity during the study. Recent white papers recommend including a NAb assay in the immunogenicity testing scheme when the protein therapeutic is highly conserved with an endogenous protein critical for a physiological function, as these results can be predictive of the potential safety impact of NAbs in humans [6, 5]. For example, preclinical studies in mice and monkeys demonstrated a direct causative relationship between neutralizing anti-TPO immunoglobulin G (IgG) antibodies and thrombocytopenia [7]. Similarly, red cell aplasia was observed in dogs administered erythropoietin and proved to be predictive of the consequences of NAbs in humans [1]. For this type of biotherapeutic, a NAb assay for detection of neutralizing antibodies against the endogenous protein may also be useful to show potential consequences of neutralization in humans.

Implementation of a NAb assay may be beneficial when a good pharmacodynamic marker and *ex vivo* drug activity assay to confirm bioactivity of circulating drug are not available [5]. In studies with a high incidence of ADA, a NAb assay may also be useful to assess if any observed toxicity is related to drug exposure, by determining whether the animals testing positive for ADAs were adequately exposed to active drug [7]. For other types of protein therapeutics, such as monoclonal antibodies (mAbs), the formation of ADAs is expected to result in minor consequences; therefore, extensive ADA characterization including NAb evaluation may not be warranted. For each program, the value of data from a NAb assay in terms of aiding in study

interpretation and predictability of potential safety risks in humans should be considered.

14.2.2. Clinical

The tiered approach to immunogenicity testing results in ADA-positive samples from the binding assay being further characterized as to neutralizing ability in either a cell-based or ligand-binding assay. Although a sample may test positive in an *in vitro* assay, the results must be interpreted in the context of the other clinical endpoints since positive results in an *in vitro* assay may not translate to clinical impact. The EMA guidance [10] suggests using bioassays for the assessment of the neutralizing capacity of antibodies; however, ligand-binding assays are also mentioned as an option.

Sample collection time points, frequency, and testing (i.e., real time, vs. at end of a specific cohort or cohorts or at the end of a study) will all be driven by the risk assessment as well as projections on drug levels at those time points. Drug may interfere in the assays and methods to remove drug (or isolate Ig) may need to be considered in order to accurately test the samples. In most cases one would make an effort to have sufficient washout samples collected based upon the half-life of the drug; however, levels of neutralizing ADA may also be diminishing during this period.

Furthermore, the EMA describes characterizing responses as either transient or chronic; hence sample collection frequency must be such that these assessments can be made. In some cases sponsors may collect many samples as part of a study but may only test a portion of them; however, the other samples are available for further testing if warranted based on preliminary results. These approaches need to be described adequately in the protocol, and it is recommended that such strategies be endorsed by regulatory agencies. It may be that the frequency of sampling may be adjusted for later phases of drug development based upon prior study experience with the incidence of antibody generation in the context of other clinical endpoints. Sample collections should be consistent across studies. That is not to say the frequency may need to be maintained, but if samples were collected monthly early on, later one may change to every other month or every three months so data at similar time points can be compared across studies. If sample collections vary across studies, the rationale for this should be explained per recommendations in the EMA guidance.

Depending upon the risk assessment, the stage of drug development at which a validated NAb assay is needed may vary. We have had requests for a validated assay as early as Phase I when there was the potential for cross-reactivity to endogenous protein, while in other situations companies have stated that they do not have validated assays until Phase III for certain monoclonal therapeutics. Further, we have been told we could not start subsequent studies until we had provided validated NAb data from an ongoing Phase II study. Thus expectations for when a validated assay is in place as well as when

data are provided to a regulatory agency may very depending upon the program and the perceived risk of a serious impact on patient safety if NAbs were to develop.

There is no "one size approach" that fits all programs, and hence the strategy for a particular program needs to be driven by risk assessment as described in Chapter 1, by Koren et al.

14.3. ASSAY FORMATS: CELL-BASED VERSUS LIGAND-BINDING IMMUNOASSAYS

There are two general formats used for the detection of neutralizing antibodies against protein therapeutics: a cell-based assay or functional bioassay; and a competitive ligand-binding immunoassay. The format for detecting NAbs may also depend on the nature of the product and associated risk [6]. Traditionally, cell-based assays are the preferred format since NAbs function by inhibiting or blocking the biologic function activity of drugs. Therefore, some feel that cell-based bioassays may provide the most appropriate biological model for the measurement of anti-drug NAbs [11].

While bioassays may be the most relevant format for agonist products, ligand-binding approaches may be acceptable and biologically relevant for detection of NAbs against antagonistic mAbs based on the mechanism of action. In fact, a draft guideline issued by the EMA on the immunogenicity assessment of monoclonal antibodies [12] states that ligand-binding assays may be the platform of choice for detection of NAbs against mAb therapeutics based on the mode of action. A disadvantage to ligand-binding assays is that they may not replicate the binding conditions on the cell surface, and adherence to plastic can alter structure, modify binding properties, and/or alter and mask epitopes. However, ligand-binding assays may allow for greater drug tolerance and sensitivity than cell-based assays.

When the drug target is a cell-based receptor, cell-based binding assays or competitive ligand-binding assays may be considered. Cell-based NAb assays should be considered for products where binding may not be representative of the biological activity of the therapeutic. Functional cell-based bioassays may also be considered for higher risk programs because of their relevance to biological function [6, 7, 11]. The following sections will review parameters to consider when developing and validating a functional cell-based NAb assay.

14.4. DEVELOPMENT OF A CELL-BASED ASSAY FOR DETECTION OF NEUTRALIZING ANTIBODIES

The function of the therapeutic protein drives the design of the cell-based NAb assay where NAbs are detected by their ability to inhibit drug activity *in vitro*. Some protein therapeutics may have more than one biological activity

(e.g., recombinant cytokines) that could be selected as the assay endpoint. For example, assays to detect NAbs against interferon beta (IFN-β) can be based on the antiviral properties of IFN or based on genes or proteins whose expression is induced by IFN-β [13]. When there is an option of multiple endpoints, the assay readout most reflective of the drug activity in patients may be considered first. However, sensitivity, precision, and the availability of reagents or instruments may also be factors to consider when selecting the assay endpoint.

In some cases, a protein therapeutic may have more than one functional domain. For example, monoclonal-based therapeutic antibodies of the IgG1 subclass bind the target via the Fab domain but also exert effector functions via the Fc region of the molecule. For this class of therapeutic, two NAb assays may be required to define the effect of neutralizing antibodies against each effector region of the molecule. Similarly, assessment of neutralizing antibody formation against enzyme replacement therapies requires at least two NAb assays, one to assess neutralization of enzyme uptake by cells and one that measures the ability to inhibit catalytic activity, as these functions are mediated by different structures in the enzyme [14]. Another example of when a second NAb assay may be useful is when the drug is a mimetic of an endogenous protein. In addition to the NAb assay against the drug, a similar cell-based NAb assay may be developed using the endogenous protein.

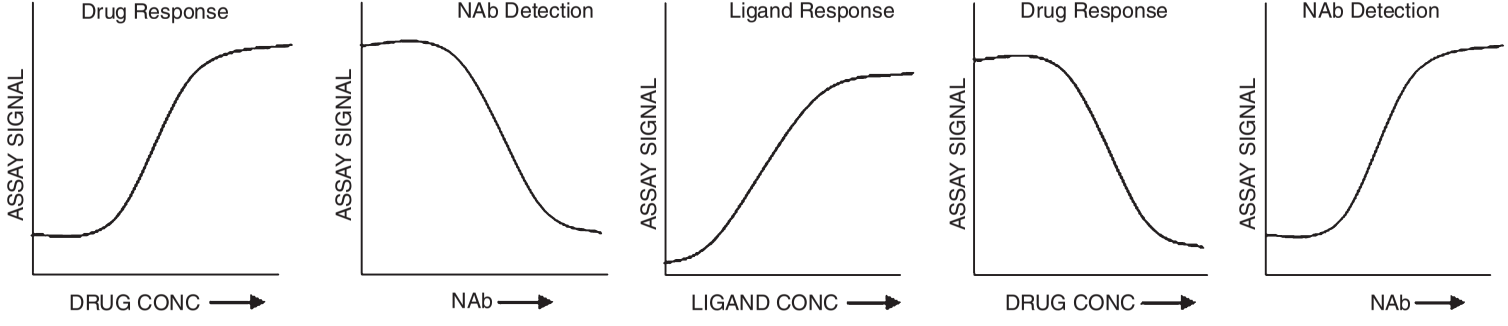
The first consideration for the NAb assay design is the mechanism of action of the drug. Protein therapeutics are generally classified as either agonist or antagonist in terms of function. Examples of agonist protein therapeutics are cytokines, growth factors, hormones, and monoclonal antibodies. Agonists function by directly binding to a target or receptor to induce the desired response. Alternatively, mAb-based therapeutics or other therapeutics that act as antagonists function indirectly by blocking a ligand from binding the target to inhibit an undesired activity. As a result, NAb assays have been classified into two general categories based on the drug's mechanism of action: direct for agonists; indirect for antagonistic drugs [11].

The assay format, direct or indirect, leads to the selection of the critical reagents required for each assay format (see Table 14.1). A direct NAb assay requires a cell line responsive to the drug, the drug, serum representative of the test sample matrix, and neutralizing anti-drug antibodies to serve as a positive control. As shown in Table 14.1, in a direct NAb assay, the cell line response correlates to the amount of drug added to the cells, resulting in a dose–response curve. When testing samples for neutralizing activity, a decrease in assay signal with cells treated with a constant concentration of drug is indicative of NAbs in the sample.

An indirect NAb assay requires a cell line responsive to ligand, drug, test species serum, and a positive control antibody (see Table 14.1). In an indirect assay, a dose–response curve is generated with ligand. Addition of drug to cells treated with a constant concentration of ligand results in signal inhibition as shown in Table 14.1. When testing samples, recovery of assay signal as shown

TABLE 14.1 NAb Assay Formats

Direct	Indirect
NAb assay format for agonistic drugs (e.g., cytokines, growth factors, hormones, mAbs)	NAb assay format for antagonistic drugs (e.g., mAbs)
Critical reagents: • Cell line • Drug • Positive control antibody • Sample matrix (e.g., serum)	*Critical reagents:* • Cell line • Drug • Ligand • Positive control antibody • Sample matrix (e.g., serum)
Assay response:	*Assay response:*



in Table 14.1 in the indirect NAb detection graph is indicative of NAbs. Considerations for the selection of the critical reagents for a NAb assay and implications for assay development and performance are discussed later in this chapter.

14.4.1. Considerations for Selection of a Cell Line

The critical selection for both formats and the basis of any cell-based assay is the choice of the cell line for the assay. The cell line selected must respond well to the protein therapeutic or ligand, to ensure appropriate sensitivity for detection of neutralizing activity. Information regarding alternate stimulants that affect the cell line in the same manner as the drug may also be useful for direct assays, to evaluate matrix interference. The cell line should yield a functional endpoint that correlates with the drug action *in vivo* and be compatible with the prospective test species. An initial option to investigate is the cell line used in the potency bioassay. The advantage of being able to use the same cell line used in the potency assay is that optimization of cell culture parameters and maintenance has already been established. Mire-Sluis [15] provides a review of bioassay formats for biotechnology products and indicates an advantage of the kinase receptor activation assay (KIRA) for immunogenicity bioassays since this format is less sensitive to the inhibitory effects of biological matrices. Alternatively, cell lines can be screened for responsiveness to the drug or ligand. A review of commonly used assay formats is provided in Table 14.2.

An important criterion when evaluating cell lines for use in a NAb assay is to select a cell line that generates an acceptable signal over noise when cells are treated with drug or ligand. The degree of response of the cell line to the drug or ligand dictates assay sensitivity. The more robust the cellular response to low concentrations of the drug or ligand, the greater is the sensitivity allowed for detection of NAbs in samples. A good starting target may be to select a cell line where at least a fivefold increase in signal is observed in response to drug or ligand treatment [11]. When considering what cell lines to screen, transfected cell lines may offer an advantage when low signal is observed with primary or natural cell lines. The drug or ligand target receptor density is usually higher in transfected cell lines, which can lead to enhanced signal. Cell lines engineered with a reporter gene linked to a promoter region induced upon drug or ligand binding also offer advantages over natural or transfected cell lines [16]. Reporter-gene assays are typically shorter in duration due to the early endpoint, are specific, may be more robust, and may offer improved assay precision versus using transfected cell lines [15].

Additional technologies have also been introduced resulting in cell lines yielding higher signal-to-noise ratios. For example, assays for the signaling of G-protein-coupled receptors (GPCRs) typically suffer from low signal-to -noise ratios [17]. However, the development of cell lines engineered to express GPCRs together with an enzyme complementation reporter system resulted

TABLE 14.2 Cell-Based Assay Formats Used for the Detection of NAbs

Method	Endpoint(s)	Advantages	Disadvantages	References
Cell line based assay	Early • Cell–receptor binding • Protein expression • mRNA expression Late • Cell proliferation	• Transfected cell lines result in higher receptor cell density, enhancing assay signal	• Requires a clonal cell line responsive to drug or ligand with sufficient signal-to-noise ratio • Longer assay time (1–3 days) • Later endpoints may result in decreased sensitivity (μg/mL versus ng/mL)	22, 23, 24, 25
Reporter gene assay	Reporter gene expression (e.g., luciferase)	• Shorter assay time (3–4 hours)	• Requires reporter gene transduced cell line	26, 21
Kinase receptor–based assay (KIRA)	Receptor tyrosine phosphorylation	• Less prone to inhibitory affects of biological matrices • Transfected cell lines result in higher receptor cell density, enhancing assay signal	• Requires kinase receptor transduced cell line and anti-phosphotyrosine antibodies • Longer assay time (24 hrs +) • Limited use to cytokine, growth factors, or other protein therapeutics possessing an integral enzyme in its receptors • Question how transfected receptor phosphorylation relates to overall biological activity	27, 28
Enzyme complementation—based assay	Enzyme complementation resulting in chemiluminescent signal	• Robust signal • Shorter assay time (hours)	• Requires engineered cell line with substrate system	18, 19
CDC assay	Lysis of target cells	• Shorter assay time	• May require sample pretreatment in some patient populations — Incomplete ADA recovery — Low throughput	29

in a greater signal-to-noise ratio upon drug binding [18]. While previously it was difficult to develop a NAb assay for drugs targeting GPCRs due to the limited signal range with drug, this technology enabled development of a cell-based NAb assay for a peptide-based therapeutic with good signal-to-noise ratio [19]. Enzyme complementation can be used for other protein targets as well where robust signals cannot be achieved with existing cell lines.

Another consideration for cell line selection is identifying a cell line that results in signal with acceptable precision. Cell-based assays typically have higher variability than immunoassays due to cell culture conditions [20]. Established cell lines that can be maintained over multiple passages with similar receptor expression are preferred over the use of primary cells. Also, cell-based assays with late endpoints (e.g., cell proliferation, protein expression) may take place over days, which may contribute to decreased precision versus assays that can be completed in hours. Precision may be improved by using a reporter cell line or other engineered cell line with an early readout. However, if a later endpoint is most appropriate for the NAb assay under development, several techniques described later in this chapter may be explored in order to improve consistency and decrease the variability due to the cells used in the assay.

A technique has been developed in which cells are cultured to a constant passage number, treated with an antimitotic drug, and frozen, enabling direct use of these cells in the assay upon thawing without further cell cultivation. The use of division-arrested cells reduces variability associated with cell division during the assay. Division-arrested frozen cells have been used to detect anti-IFN NAbs demonstrating precision and reduced assay time [21]. Pfizer Global Research and Development has also evaluated using frozen cells generated from cells at the same passage number versus cells in continuous culture in NAb bioassays and observed improved precision and consistency (unpublished data). The use of frozen cells and division-arrested cells offers convenience, shorter assay time, control over variation due to differences in cell conditions, and the potential for improved consistency if methods are performed in multiple laboratories. These techniques may be considered for all cell-based assays to decrease the assay time, improve assay precision, and provide consistency and standardization of the assay.

The effect of test species serum on the cellular response may also be assessed during selection of the cell line. Ideally, the change in cellular response to the drug and/or ligand will not change significantly in the presence of test species serum. Matrix interference may be evaluated by testing multiple individual or pooled normal serum and any relevant disease state serum in the assay to compare the cellular response in the presence and absence of serum. If matrix interference is observed with the selected cell line, there are several options to consider to overcome this issue. A matrix interference assay may be developed to discriminate positive results due to NAbs versus nonspecific serum factors, or sample pretreatment methods may be evaluated to purify the immunoglobulins prior to testing in the NAb assay. Matrix interference will be discussed further as part of assay optimization.

14.4.2. Considerations for Selection of an Endpoint

Another consideration when selecting the cell line is the biological readout of the assay, or endpoint. When selecting an assay endpoint relevant to the drug action, it is helpful to understand not only the binding target of the drug or ligand but also what genes and proteins are induced by the drug or ligand binding to its target. Assay endpoints can be categorized by early (e.g., mRNA, nuclear events) or late (e.g., cell proliferation, expression of cellular markers or enzymes) events. Gupta et al. [11] reviewed common assay endpoints for NAb assays.

Earlier endpoints may offer improved precision and specificity over later endpoints, as early endpoints such as nuclear events are often restricted to drug or ligand binding while later endpoints such as apoptosis may be activated by other nonspecific factors present in samples. Using earlier endpoints often results in shorter assay times as well. However, late endpoints may be more biologically relevant. Development of reporter-gene assays that identify activation of genes involved in drug or ligand function may be more precise, be shorter, take hours versus days to perform, and appear less affected by extraneous agents [15]. Lam et al. [26] reported validation of a luciferase reporter gene assay to measure NAbs to IFN-β that was comparable to the standard cytopathic effect (CPE) assay for NAbs and offered reduced assay time and appropriate sensitivity and precision. Kinase receptor activation assays (KIRA) also allow for rapid measurement of neutralizing activity and are also less prone to interference from biological matrices [27, 30]. The robustness of the KIRA format in the presence of serum and the observed specificity may be attributable to the assay endpoint, one of the first steps in receptor signaling after ligand binding [27]. A disadvantage to gene reporter, KIRA and enzyme complementation assay formats is the need for engineered cell lines, which may not always be readily available and may also be costly. In addition, the KIRA also requires a specific anti-phosphotyrosine antibody. Advances in cell engineering and technologies offer cell lines with endpoints yielding improved signals, sensitivity, precision, and reproducibility and may enable cell-based functional analysis for NAbs that may not have been possible previously. However, selecting engineered cells lines and endpoints requiring special instrumentation may increase the cost of analysis.

In cases where the therapeutic has antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) activity, it may be appropriate to have a NAb assay based upon this activity. McCutcheon et al. [29] report the utilization of a CDC assay with a sample pretreatment step to evaluate NAb activity in rheumatoid arthritis patients who received an IgG1 mAb with CDC activity.

The choice of format also depends on whether the reported results will be qualitative versus quasi quantitative. Qualitative assays test at a single dilution where assay matrix interference is minimal [i.e., minimum required dilution (MRD)], which allows for higher throughput. Quasi-quantitative assays generally report titers or amount of drug neutralized, which requires multiple

dilutions of samples and generally is not as high throughput. This data may be useful to assess what NAb levels may be related to changes in surrogate markers or clinical observations. A quasi-quantitative analysis may reveal that changes in safety or efficacy correlate to a specific titer or a threshold amount of drug neutralized, above which clinical effects are observed and below which no impact of NAbs on clinical observations are observed. For example, high NAb titers against IFN-β products have been associated with reduced efficacy and greater clinical impact in multiple sclerosis patients [4, 31, 32].

14.4.3. Considerations for Drug and Ligand

The drug used in the assay should be representative of the drug to be administered in the study, to ensure the assay is capable of detecting antibodies to the administered drug. During the course of drug development, changes or improvements may be made to the drug manufacturing process that may result in a different amount of protein glycosylation, changes in process-related impurities, or a different formulation. These types of changes may impact the immunogenicity of the drug [1]. Therefore, to ensure antibodies elicited against the administered drug are measured in the NAb assay, it is recommended that the drug material used in the assay is manufactured and formulated via the same process as the drug to be administered in the study. Exceptions may be made when the drug is conjugated to a carrier such as polyethylene glycol (PEG). In this case, the unconjugated form of the drug may be used in the assay to minimize nonspecific effects on the cells.

When the protein therapeutic under development is similar to other marketed drugs, it may be useful to consider using a World Health Organization (WHO) International Standard, if available, to allow for standardization. For example, an International Standard has been established for IFN products and allows for calibration of IFN NAb assays across labs and companies [33]. Other approaches to standardization will be reviewed in a subsequent section.

For ligands used in indirect assays or in assays to detect NAbs against endogenous proteins, use the native protein if available or a recombinant form as an alternative.

14.4.4. Considerations for Controls

A neutralizing anti-drug antibody positive control is required to optimize assay parameters and to assess the relative sensitivity during validation. During routine testing, controls can be used to monitor assay performance. If the positive control in the screening assay is also neutralizing than this antibody could also be used as the positive control in the NAb assay. If the positive control is a mAb, then using the same antibody in the screening and NAb assay offers the advantage of being able to compare the relative sensitivity between the assays. Since an affinity purified polyclonal antibody may include

TABLE 14.3 Controls for Direct and Indirect NAb Assays

Control	Direct	Indirect
Background	Cells	Cells
Maximal signal	Cells + drug	Cells + ligand
Drug activity	Cells + drug	Cells + ligand + drug
NAb positive control (PC)	Cells + drug + NAb PC	Cells + ligand + drug + NAb PC

non-neutralizing antibodies, this prevents such a comparison. If this comparison indicates that the NAb assay may be more sensitive than the binding assay, then sponsors may consider testing all samples in the NAb assay for high-risk products [6]. For clinical assays, a positive control may also be antisera from hyperimmunized animals or humans, if available. In many cases, hyperimmunized serum representative of the species to be evaluated may not be available. Therefore, neutralizing polyclonal antibodies from another species or monoclonal antibodies may be developed against the drug for use as a positive control. If neutralization cannot be achieved with a single mAb, then a mixture of mAbs may need to be used as a control. It may also be useful to have NAbs of varying affinities to better characterize the detection capabilities of the assay. However, as for any assay, the positive control does not necessarily reflect the antibodies present in actual patient samples, which may differ in terms of types of antibodies, affinity, and avidity.

Controls to consider implementing for NAb assays are listed in Table 14.3. Controls are useful to evaluate the interactions between assay components. In direct NAb assays, the critical reagents include cells, drug, and a positive control. A control consisting of cells only provides an indicator of assay background and any nonspecific cell response due to sample matrix. A control to monitor the maximum cellular response is also useful, to evaluate the consistency of drug activity between assays. Routinely calculating the fold increase in signal with drug over the signal with cells alone allows for monitoring of cell culture quality and drug activity [11]. Monitoring overall assay performance may be conducted by including a control with cells, drug, and the positive control antibody. Changes in control performance may indicate a loss of cell or drug activity or matrix interference and may require further investigation.

In an indirect NAb assay, the critical regents include cells, ligand, drug, and a positive control. Controls consisting of cells alone and cells with ligand allow for monitoring of the assay background signal and the maximal assay signal, respectively. These controls are useful to verify the activity of the ligand and cell culture. In addition, a control of cells only allows for evaluation of matrix interference. To monitor drug activity, a control may also be included consisting of cells, ligand, and drug. Overall assay performance may be monitored by including a control with cells, ligand, drug, and the positive control antibody.

Because indirect assays tend to be more complex than direct assays due to the additional component, an additional control is required to monitor the interactions between all assay reagents. As discussed previously these controls allow for the monitoring of reagent and assay performance between assays and over time.

14.5. OPTIMIZATION OF A CELL-BASED ASSAY FOR DETECTION OF NEUTRALIZING ANTIBODIES

Once the critical assay reagents have been selected, the reagent concentrations and assay conditions should be optimized to further characterize assay performance as well as to define assay parameters for validation. Parameters to evaluate for optimal assay performance include drug or ligand concentration, incubation conditions, washing or lysing conditions, and cell density and are reviewed in Gupta et al. [11]. In addition optimization parameters to be considered for plate-based assays may include the effect of plate color (for luminescent assay), plate configuration, and duration of plate shaking. Conditions should be selected that yield optimal signal-to-noise ratios. Parameters may be optimized one at a time, but the recommended approach to optimization is to use a factorial design approach with consultation from a biostatistician so that interactions between assay parameters may be incorporated in the analysis. A few parameters with specific significance to cell-based NAb assays are discussed in the following subsections.

14.5.1. Drug–Ligand Concentration

Selection of the drug or ligand concentration in a direct or indirect NAb assay, respectively, has a significant impact on the assay's relative sensitivity. A good starting point is to evaluate concentrations within the linear range of the dose–response curve ($EC_{50–80}$ range). To allow for detection of NAbs, it is recommended that the selected concentration result in at least a fivefold increase in signal [11]. The drug or ligand concentration dictates assay sensitivity and the signal-to-noise ratio resulting from cells treated with drug or ligand may be incorporated as a control in each assay to evaluate assay performance.

In an indirect assay, following selection of the optimal ligand concentration, evaluation of the drug-induced inhibition of the cellular response may be useful to select the optimal drug concentration in the assay. The drug concentration selected for indirect assays also determines the assay relative sensitivity. For example, if the drug concentration is in excess of the amount required to inhibit the ligand, then a greater amount of NAbs will be required to neutralize the drug and restore signal in the assay. Therefore selection of the lowest level of drug required to inhibit the ligand may offer increased sensitivity.

14.5.2. Matrix Interference

Factors present in serum samples can affect cells in a stimulatory or inhibitory fashion, resulting in a loss of assay specificity [11]. In addition, cell lines are often pleiotropic and may respond to multiple related cytokines or agents in matrix, so specificity of cell line should be evaluated during development and be considered when selecting a cell line. For example, disease state samples may have elevated cytokine levels that result in assay signal in an indirect NAb assay mimicking NAb action and causing a false positive result. Other factors present in samples that may interfere in a NAb assay include soluble receptors, drug, endogenous homologue, concomitant medications, lipids, and complement. Soluble receptors present in samples may also lead to false positive results by binding drug or acting directly on cells. The potential for matrix interference should be assessed during method development by testing multiple individuals or pooled normal serum and any relevant disease state serum, as disease state sera may contain additional factors. Because sera from different vendors may perform differently, it is recommended to test serum from multiple vendors when evaluating matrix interference. Another caution is that commercial or control sera may not mimic increases in soluble factors observed when patients are treated with the drug. In some cases, the target may increase up to 100-fold in treated patients, while these levels may not be present in control or commercially sourced sera. If a level of target in patients following drug treatment has been estimated or is known from previous studies, then sera may be spiked in excess to evaluate the potential for interference in the assay.

The minimum required sample dilution (MRD), should be determined to minimize interference from matrix components during testing [34]. The MRD also impacts assay sensitivity. Hemolyzed, lipemic, or incompletely clotted samples may also impact assay results and may be evaluated to assess whether these types of samples can produce reliable results in the assay.

A separate matrix interference assay may also be developed and validated in parallel with the NAb assay, to discriminate positive results due to NAbs present in the sample versus false positive results due to nonspecific serum factors. Gupta et al. [11] review the development and validation parameters for matrix interference assays. The matrix interference assays include the alternative stimulus format for direct assays, sample-induced response format for indirect assays, and immunoglobulin depletion methods that apply to direct and indirect assays. In practice, Wei et al. [24] developed a cell-based assay for the detection of NAbs against administered recombinant human erythropoietin (rhEPO) based on a murine IL-3-dependent 32D cell line transfected with human EPO receptors. This cell line responds to human EPO and murine IL-3 with proliferation allowing for development of an alternative stimulus assay to evaluate matrix interference. Samples from patients administered EPO were tested for their ability to inhibit EPO and mIL-3-induced proliferation in parallel. In this testing scheme, to report a sample as positive for

drug-specific NAbs, the sample would test positive with drug stimulus in the direct NAb assay and negative when the alternate stimulus, mIL-3, was used in the assay. One to five percent of samples tested demonstrated non-specific inhibitory effects [24].

14.5.3. Cell Parameters

As mentioned earlier, much of the inherent variability in cell-based assays is attributable to the cells used during testing. Therefore, the conditions of the cells during testing, cell culture parameters and media should be well defined for optimal and consistent assay performance. In terms of media optimization, assay robustness may be impacted by different lots and sources of media components and fetal bovine serum [25]. Therefore, when possible, it may be useful to test different lots and sources of media components to assess the impact on assay performance. Also, when the drug is a peptide-based therapeutic, the inclusion of protease inhibitors in the media may be required if the peptide is susceptible to endogenous serum proteases [19].

The condition of cells at the time of testing, in terms of cell density, viability, and cell cycle stage, may impact assay performance and variability. Cell lines can lose biological responsiveness over time, so it is suggested to have a well-characterized cell bank and know how long the cells can be passaged before the response is compromised. In the EPO NAb assay with a cell proliferation endpoint, if the cells were overgrown, they lost dependence on rhEPO for growth, leading to higher background [24] and demonstrating the correlation between cell density and assay performance. In addition, the cell passage number can affect assay variability, as decreases in assay precision have been observed as the cell passage numbers increase [11]. Therefore, it is important to define the cell culture parameters, including acceptable number of cell passages for cells to be used in the NAb assay to ensure consistent and reliable assay performance.

14.5.4. Drug Interference

Drug present in patient samples may interfere in the NAb assay, causing false negative results. Drug present in samples may interfere by increasing the assay signal or by complexing with NAb and limiting its detection. Ideally, drug interference can be avoided by collecting samples when the drug concentration is low or has been eliminated from circulation by collecting samples following a period when no drug is administered. For some programs this may not be possible and may be especially problematic for monoclonal-based therapeutics due to their long half-lives and high doses. Several different methods to overcome drug interference have been applied in NAb assays. Similar to sample pretreatment methods described for screening ADA assays, acid dissociation followed by removal of excess drug by solid-phase adsorption

has allowed for detection of NAbs in samples containing high levels of therapeutic [35]. An alternate method proposed for testing samples with high levels of drug is a drug quantitation based approach n which samples are tested with and without exogenously added drug [27]. Any sample pretreatment methods utilized for NAb testing require optimization and validation prior to use for routine testing. For example, optimization or validation parameters for an acid pretreatment procedure as described by Lofgren et al. [35] may include studies to prove that NAbs remain active following acid treatment and to determine the optimal pH and duration of acid treatment.

Increased ligand or target levels in samples may also interfere in indirect NAb assays, potentially leading to false positive results. Typically in indirect assays, NAbs are detected by binding the drug in the assay, freeing the ligand to bind to the cellular target and initiate a cellular response. However, if additional ligand is present in samples, the increased concentration of ligand may restore assay signal even without NAbs present, leading to a false positive result. If necessary, sample pretreatment methods to minimize the ligand levels in samples may be explored. One method to evaluate may be to remove the ligand from samples by incubating the sample with an anti-ligand-specific antibody attached to beads or other solid-phase matrix. Following removal of the solid-phase matrix, the ligand-depleted samples may be tested in the NAb assay. Size exclusion methods may also be considered for removal of ligand from samples. Alternatively, all immunoglobulins could be purified from samples to remove all matrix factors.

14.6. VALIDATION OF A CELL-BASED ASSAY FOR DETECTION OF NEUTRALIZING ANTIBODIES

The objective of validation of a method is to demonstrate that it is suitable for its intended purpose [36]. Therefore, validation of a cell-based assay to detect NAbs against a protein therapeutic must demonstrate that the assay has the appropriate accuracy, precision, sensitivity, specificity, and robustness to reliably and reproducibly detect low amounts of drug-specific NAbs in complex biological matrix. Currently, there is no published formal guidance to describe the validation parameters for a NAb assay. The Ligand Binding Assay Bioanalytical Focus Group (LBABFG) of the American Association of Pharmaceutical Scientists (AAPS) is writing a white paper to address validation of cell-based NAb assays. While there are guidance documents covering analytical validation of quantitative assays (ICH, FDA, US Pharmacopeia) not all parameters apply to NAb assays. Some validation parameters described in white papers for validation of ADA screening assays may also be applicable to the validation of NAb assays [34, 37]. Considerations regarding key validation parameters specific to cell-based NAb assays are discussed in the following subsections.

14.6.1. Controls

During development and optimization, neutralizing anti-drug antibodies should have been identified to serve as positive control(s) in the assay. Positive controls are used during validation to evaluate precision, set assay acceptance criteria, and define relative sensitivity. The positive control may be tested at a high and low (near the cut point) concentration or be titrated to report a titer. A negative control, usually a pool of serum representative of test samples, should also be included in each validation assay as well as assays conducted as part of routine testing. During routine testing the controls are used to determine if the assay performed as expected by determining if assay acceptance criteria were met. Assay acceptance criteria are typically set around the controls established for the assay. Additional controls unique to direct and indirect cell-based NAb assays are listed in Table 14.3. The addition of a non-neutralizing positive control may also be considered as an additional control for false positives. All control data generated during validation should be used to set assay acceptance criteria. Control data can continue to be monitored against acceptance criteria during testing, to ensure the assay continues to perform as expected. Any "drift" in control data over time may indicate a change in assay performance. Shankar et al. [37] advises revalidating assays as needed to reduce assay "drift."

14.6.2. Cut Point

The cut point is used to define results as putative positive versus negative for neutralizing activity. To ensure a robust data set for the cut point analysis, it is recommended to incorporate biological and assay variability into the cut point study by testing serum from multiple individuals, testing the samples over several days, and including more than one analyst if appropriate [34, 37]. One recommendation is to test a panel of 20–50 individual serum samples representative of test samples and sourced from multiple vendors at the MRD to determine the background signal [11]. The data set may be analyzed for outliers and the results can be used to calculate a fixed, floating, or dynamic cut point as described by Shankar et al. [37]. The type of cut point used depends on assay variability as well as variability in sample background results.

For a direct assay, the cut point would be set at the lower confidence limit so that samples testing below the cut point would be reported as positive for neutralizing activity. Samples with signal at or above the cut point would be reported as negative for NAbs. For an indirect assay, the cut point would be set at the upper confidence limit based on the drug's inhibitory role in the assay. In an indirect assay, samples with signal greater than the cut point indicate the presence of NAbs blocking the drug and restoring the signal in the assay and would be reported as positive. Cut points may need to be re-established, as clinical studies may be performed in different patient populations.

14.6.3. Sensitivity

Relative assay sensitivity may be estimated by calculating the concentration of the positive control detected at the assay cut point. A study design for the determination of relative sensitivity incorporating assay variability is reviewed by Shankar et al. [37]. While this determination of assay sensitivity is relative since the positive control may not be representative of actual test samples, regulatory authorities will expect a definition of what dilution of the positive control is required to neutralize a specified amount of the biological activity of the drug product or endogenous counterpart [38]. The intent of a NAb assay is to detect all clinically relevant NAbs, and therefore the goal of validation is to demonstrate the assay is sensitive enough to detect clinically relevant NAbs. It is unlikely to be able to correlate the level of NAb required to neutralize the drug *in vitro* to the amount required to neutralize the drug *in vivo*. Correlation of NAb results with alterations in pharmacokinetics, pharmacodynamic markers, or clinical observations should provide evidence that the assay's sensitivity is acceptable.

14.6.4. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components expected to be present in the test sample [36]. Validating the specificity of the assay ensures that reported positive results are due to the presence of NAbs in the samples and not a result of other components in the sample mimicking NAb activity. Some considerations for specificity assessment are listed as follows. Is the cell line response specific to drug and/or ligand or is the cell line pleiotropic, reactive to multiple factors? Are there other factors that may be in the serum that could mimic NAbs by binding and inhibiting drug activity (e.g., soluble target or receptor)? A lack of specificity may lead to false positive or false negative results. A false positive result may be generated in a direct NAb assay when other serum components such as soluble receptors block drug activity resulting in a decreased assay signal indicative of NAbs. In an indirect NAb assay, if other factors are present in test samples capable of generating a signal in the assay in the absence of NAbs, then a false positive result may be reported. Specificity may be routinely evaluated for each test sample by testing each sample in a matrix interference assay (e.g., alternative stimulus assay, immunoglobulin depletion) as described by Gupta et al. [11] in addition to testing in the NAb assay.

14.6.5. Drug Interference (Selectivity)

As discussed in the optimization section, bioassays may fail to detect NAbs in the presence of high drug levels, causing false negative results. Drug present in samples may complex with NAbs, limiting detection, or may decrease the relative sensitivity of the NAb assay due to the increase in drug levels in the

assay. Ideally, test samples will be collected following a washout period so that drug levels will be minimized, allowing for evaluation of NAbs in the assay with minimal drug interference. Samples collected during the dosing phase of the study should be collected predose to minimize the drug levels in samples. However, if drug is expected to be present in samples, acid pretreatment methods to dissociate drug and antibody complexes [35] or a drug quantitation based testing approach [27] may be considered. To estimate what levels of drug may interfere in the assay, also referred to as drug tolerance, varying concentrations of drug may be spiked into the positive control during validation of the assay. Similar to the determination of assay sensitivity, the assessment of drug tolerance is based on the characteristics of the positive control antibody, which may not be representative of samples. Therefore, the drug tolerance limit should not be directly applied to test samples to predict what drug levels will interfere with NAb detection in samples.

14.6.6. Precision

Precision studies estimate the variability within an assay (intra-assay or repeatability) and between assays (inter-assay variability). Inter-assay precision studies should incorporate expected within-lab variations such as days, reagent lots, instruments, analysts. Precision results are typically reported as a percentage coefficient of variation. The acceptance criterion is usually expressed as a coefficient of variation or a titer range and depends on the technology used to read the assay endpoint and on assay methodology, and it should be defined based on assay capability and intended use. Refer to Shankar et al. [37] for recommendations regarding study designs and analysis for precision experiments. While samples may typically be tested in duplicate, sponsors may consider increasing the number of replicates if necessary in order to achieve desired precision. Also, the plate configuration, including control and sample placement, may be optimized to reduce any potential plate position effects such as avoiding use of the outer wells when increased variability is observed in this subset of wells.

14.6.7. Stability

Because of the tiered testing strategy for immunogenicity, the freezing and thawing of samples may be unavoidable. Therefore, the impact of freeze–thaw on sample stability may be evaluated to support the expected sample storage conditions. While the positive control can be tested for stability, the results would not necessarily reflect study sample stability. Shankar et al. [37] state that the stability of ADA can be approximated by the stability of serum or plasma immunoglobulin to any antigen. Therefore, available clinical and nonclinical samples may be characterized separately for stability and the results may be extended to other programs. The impact of potential freeze–thaw cycles per sample may be minimized by making multiple sample aliquots. The

expected storage conditions such as the number of freeze–thaw cycles, storage temperature, and length of storage may be considered when designing the stability testing plan.

All critical reagents in the assay may be considered for stability testing during validation to document their stability under the expected storage conditions to support routine testing. Any significant changes in assay signal may indicate a loss of stability.

14.6.8. Robustness

Robustness is the capability of the assay to remain unaffected by small variations in assay procedures [36]. The level of testing depends on use of the assay. For instance, if the assay is used to support long clinical trials and postmarketing testing, where testing may be done at multiple laboratories over multiple years, then more rigorous testing may be warranted versus an assay used to support a short nonclinical study. To streamline testing it is recommended to work with a biostatistician to use a multifactorial design of experiments (DOE) that incorporates interactions between assay parameters [34].

14.6.9. Analysis and Reporting Results

Results for quasi-quantitative assays may be expressed as a titer or the amount of drug neutralized per milliliter of serum. If results are reported as the amount of drug neutralized per milliliter of serum, the assay also includes a full drug curve, as sample results are interpolated against the drug standard curve to determine the amount of drug detected. For this analysis, the interpolated result is then subtracted from the fixed amount of drug added to the sample and multiplied by the sample dilution factor to determine the concentration of drug in the patient. A potential disadvantage of this method is that if sample results are not parallel to the standard drug response curve, then results may be less accurate. This type of analysis would also require additional validation parameters such as sample parallelism, precision of the drug standard curve, and linearity of the drug curve.

For assays where variability is observed in baseline responses between patients, an alternative assay analysis may be considered based on a shift in the EC_{50} post-pretreatment and reporting results qualitatively. For this type of analysis, drug would be added at multiple concentrations to samples. An increase in the EC_{50} is indicative of NAbs.

To facilitate the analysis and interpretation of ADA data by sponsors and regulatory agencies, Ponce et al. [5] recommend presenting ADA data with relevant exposure data and key pharmacodynamic (PD) marker(s) per individual test subject over time in preclinical studies. This type of data presentation enables an evaluation to detect possible correlations between an individual's ADA status (e.g., NAb positive versus negative) and any changes in drug exposure or toxicity. Similarly, ADA data from clinical studies may be

reported with the associated drug vial and batch numbers, pharmacokinetic (PK), and safety data per patient to aid in study interpretation and regulatory review. Therefore, sponsors may want to consider presenting NAb assay results for preclinical and clinical study reports in an integrated data summary table including ADA, PK, PD, and safety data per individual over time.

14.7. STANDARDIZATION

At present, standard assays are not typically available across a product class. The use of different antigens (drug or ligand) at different concentrations combined with differences in cell lines, endpoints, and analysis procedures makes it difficult if not impossible to compare results between products, laboratories, sponsors, or even across studies. Differences in assay formats, reagents, and conditions result in differences in assay sensitivity, specificity, and precision. Therefore, it is generally not possible to compare immunogenicity rates of two products in the same class unless the assay is done by a third party using an assay validated as equally sensitive and specific for detecting immune responses to both products. This issue is noted in a drug's prescribing information to inform patients that comparison of the incidence of ADAs to other products may be misleading because the reported incidence of ADAs is dependent on factors such as assay sensitivity, specificity, sample handling, and methodology. The benefits of standardization of NAb assays include the ability to compare NAb incidence across products within a class and to allow clinicians to better understand the correlation between NAb development and clinical effects.

Significant progress has been made in establishing a standard cell-based assay for detection of NAbs against interferon-beta (IFN-β) products of which there are three registered products. The need for a standardized NAb assay for IFN-β products was identified because the NAb assay format was recognized as a key component contributing to the variability of reported NAbs in patients [1]. The published assay formats for detection of NAbs to IFN-β include an antiviral cytopathic assay (CPE), myxovirus resistance protein A (MxA) protein assay, MxA mRNA assay, and a luciferase gene reporter assay [13, 21–23, 26]. The lack of standardization across methods led to an inconsistent message on the impact of NAbs. An immediate need existed for standardization of these assays to facilitate effective clinical management of patients treated with IFN-β [13]. The goals of a common assay were to harmonize antibody sections for each product by expressing titers and incidence rate based on a common assay and to correlate titers obtained by the common assay with clinical effects [33]. The consensus assay format was based on the measurement of MxA protein as the endpoint, and the standardization process included developing a uniform protocol including identical sourcing of cells and reagents. To ensure the method was reproducible across manufacturers and testing sites, a blind panel of sera was tested in each laboratory to demonstrate comparability of results between sites [33]. The processes used to

standardize the cell-based NAb assay for IFN-β products may be applicable to standardization of other cell-based NAb assays.

A key aspect of standardization of a cell-based NAb assay is to identify a source of critical reagents (e.g., cell line, antibodies, and drug or ligand) capable of supplying all test sites, as variations in critical reagents may impact assay performance. Also, the use of division-arrested cells as described by Lallemand et al. [21] may reduce the variability associated with cell culture parameters during the assay, providing increased consistency and reproducibility of assay results across test sites. Sourcing identical reagents and identifying techniques to limit assay variability are a few parameters to consider when standardizing a cell-based NAb method. Comparability of results across test sites may be evaluated by testing the same panel of samples at all test sites. Standardization of methodologies to detect anti-drug NAbs may be useful to compare the incidence of NAbs and the clinical impact across products of the same class.

14.8. CONCLUSION

Neutralizing antibodies developed against protein therapeutics may impact the safety and/or efficacy of a therapeutic drug. NAbs against protein therapeutics may be assessed using ligand-binding or cell-based assays. Cell-based assays may provide the most appropriate biological model for measuring the neutralizing capacity of ADAs and are traditionally the preferred assay format. The expectations for when a validated NAb assay should be in place to support drug development vary depending on the program and the perceived risk of NAbs to patient safety. This chapter has outlined strategies and key performance parameters for the development, optimization, and validation of cell-based assays to measure NAbs. It is critical to have a validated assay that can detect clinically relevant events that may be related to NAbs. Methods to standardize the NAb assay may also be of value when the drug under development is similar to an already marketed protein therapeutic. Standardization of a NAb assay across a product class as was described for IFN products would allow for comparison of NAb occurrence and any associated clinical impact across products.

REFERENCES

1. Schellekens, H. (2002). Bioequivalence and the immunogenicity of biopharmaceuticals. *Nature Rev Drug Discov*, *1*, 457–462.
2. Li, J., Yang, C., Xia, Y., et al. (2001). Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood*, *98*, 3241–3248.
3. Casadevall, N., Nataf, J., Viron, B., et al. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.

4. Cohen, B.A., Oger, J., Gagnon, A., et al. (2008). The implications of immunogenicity for protein-based multiple sclerosis therapies. *J Neurol Sci*, *275*, 7–17.
5. Ponce, R., Abad, L., Amaravadi, L., et al. (2009). Immunogenicity of biologically-derived therapeutics: assessment and interpretation of nonclinical safety studies. *Regul Toxicol Pharmacol*, *54*, 164–182.
6. Shankar, G., Pendley, C., Stein, K.E., et al. (2007). A risk-based bioanalytical strategy for the assessment of antibody immune responses against biological drugs. *Nature Biotechnol*, *25*, 555–561.
7. Koren, E., Smith, H.W., Shoers, E., et al. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333*, 1–9.
8. Schneider, C.K., Papaluca, M., Kurki, P. (2009). A European perspective on immunogenicity evaluation. *Nature Biotechnol*, *27*, 507–508.
9. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. (2009). *Addendum to ICH S6. Preclinical safety evaluation of biotechnology-derived pharmaceuticals, October 2009*.
10. European Medicines Agency (EMA). (2007). Guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins. EMA/CHMP/BMWP/14327/2006.
11. Gupta, S., Indelicato, S.R., Jethwa, V., et al. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.
12. European Medicines Agency (EMA). (2010). Immunogenicity assessment of monoclonal antibodies intended for in vivo clinical use. EMA/CHMP/BMWP/86289/2010.
13. Deisenhammer, F., Schellekens, H., Bertolotto, A. (2004). Measurement of neutralizing antibodies to interferon beta in patients with multiple sclerosis. *J Neurol*, *251* (Suppl. 2), 31–39.
14. Wang, J., Lozier, J., Johnson, G., et al. (2008). Neutralizing antibodies to therapeutic enzymes: considerations for testing, prevention and treatment. *Nature Biotechnol*, *26*, 901–908.
15. Mire-Sluis, A.R. (2001). Progress in the use of biological assays during the development of biotechnology products. *Pharm Res*, *9*, 1239–1246.
16. Brasier, A.R., Ron, D. (1992). Luciferase reporter gene assay in mammalian cells. *Methods Enzymol*, *216*, 386–397.
17. Hammer, M.M., Wehrman, T.S., Blau, H.M. (2007). A novel enzyme complementation-based assay for monitoring G-protein-coupled receptor internalization. *FASEB*, *21*, 3827–3834.
18. von Degenfeld, G., Wehrman, T.S., Hammer, M.M., Blau, H.M.. (2007). A universal technology for monitoring G-protein-coupled receptor activation *in vitro* and non-invasively in live animals. *FASEB J*, *21*, 3819–3826.
19. Whritenour, J., O'Donnell, L., Donovan, C., Finco, D. (2009). *Validation of a bioassay for the detection of neutralizing antibodies against a biotherapeutic peptide. Poster M1044. National AAPS meeting, Seattle, WA*.

20. Meager, A. (2006). Measurement of cytokines by bioassays: theory and application. *Methods*, *38*, 237–252.
21. Lallemand, C., Meritet, J.F., Erickson, R., et al. (2008). Quantification of neutralizing antibodies to human type I interferons using division-arrested frozen cells carrying an interferon-regulated reporter-gene. *J Interferon Cytokine Res*, *28*, 393–404.
22. Gilli, F., van Beers, M., Marnetto, F., et al. (2008). Development of a bioassay for quantification of neutralizing antibodies against human interferon-beta in mouse sera. *J Immunol Methods*, *336*, 119–126.
23. Bertolotto, A., Sala, A., Caldanon, M., et al. (2007). Development and validation of a real time PCR-based bioassay for quantification of neutralizing antibodies against human interferon-beta. *J Immunol Methods*, *321*, 19–31.
24. Wei, X., Swanson, S.J., Gupta, S. (2004). Development and validation of a cell-based bioassay for the detection of neutralizing antibodies against recombinant human erythropoietin in clinical studies. *J Immunol Methods*, *293*, 115–126.
25. Baltrukonis, D., Finco-Kent, D., Poirier, M., et al. (2006). Development and validation of a quasi-quantitative bioassay for neutralizing antibodies against CP-870893. *J Immunotoxicol*, *3*, 157–164.
26. Lam, R., Farrell, R., Aziz, T., et al. (2008). Validating parameters of a luciferase reporter gene assay to measure neutralizing antibodies to IFNβ in multiple sclerosis patients. *J Immunol Methods*, *336*, 113–118.
27. Hu, J., Gupta, S., Swanson, S.J., et al. (2009). A bioactive drug quantitation based approach for the detection of anti-drug neutralizing antibodies in human serum. *J Immunol Methods*, *345*, 70–79.
28. Pennucci, J.U., Swanson, S., Kaliyaperumal, A., Gupta, S. (2010). Multiplex evaluation of a cell-based assay for the detection of antidrug neutralizing antibodies to panitumumab in human serum using automated fluorescent microscopy. *J Biomol Screen*, *15* (6), 644–652.
29. McCutcheon, K., Quarmby, V., Song, A. (2010). Development and optimization of a cell-based neutralizing antibody assay using a sample pre-treatment step to eliminate serum interference. *J Immunol Methods*, *358*, 35–45.
30. Sadick, M.D., Intintoli, A., Quarmby, V., et al. (1999). Kinase receptor activation (KIRA): a rapid and accurate alternative to end-point bioassays. *J Pharm Biomed Anal*, *19*, 883–891.
31. Perini, P., Calabrese, M., Biasi, G., Gallo, P. (2004). The clinical impact of interferon beta antibodies in relapsing-remitting MS. *J Neurol*, *251*, 305–309.
32. Polman, C., Kappos, L., White, R., et al. (2003). Neutralizing antibodies during treatment of secondary progressive MS with interferon β-1b. *Neurology*, *60*, 37–43.
33. European Medicines Agency (EMA). (2008). Beta-interferons and neutralizing antibodies (in multiple sclerosis). EMA/CHMP/BWP/580136/2007.
34. Mire-Sluis, A.R., Barret, Y.C., Devanarayan, V., et al. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods*, *289*, 1–16.
35. Lofgren, J.A., Wala, I., Koren, E., et al. (2006). Detection of neutralizing antitherapeutic protein antibodies in serum or plasma samples containing high levels of the therapeutic protein. *J Immunol Methods*, *308*, 101–108.

36. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. (2005). Q2(R1). Validation of analytical procedures: text and methodology.
37. Shankar, G., Devanarayan, V., Amaravadi, L., et al. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48*, 1267–81.
38. Chamberlain, P. (2008). Presenting an immunogenicity risk assessment to regulatory agencies. In *Immunogenicity of Biopharmaceuticals*. van de Weert M, Moller EH, eds. Springer, New York, 239–258.

13

STANDARDIZATION AND VALIDATION OF IMMUNOASSAYS

Daniel Kramer

13.1. INTRODUCTION

Recombinant biopharmaceuticals are widely used in clinical medicine, in particular for the treatment of chronic diseases such as certain forms of cancer and various forms of inflammatory and autoimmune diseases. Repeated administration of biopharmaceuticals can result in a loss of immune tolerance, leading to an immune response and the production of anti-drug antibodies (ADAs). Although ADAs are often without any apparent untoward consequences, in some cases ADAs can adversely affect drug pharmacokinetics, pharmacodynamics, bioavailability, and efficacy and may even lead to immune complex disease, allergic reactions, and in some cases severe autoimmune reactions [1, 2].

Assessment of immunogenicity is important for the interpretation of pharmacokinetic data in preclinical studies and is an essential part of drug safety evaluation in clinical studies. A number of white papers have outlined a general strategy for the assessment of immunogenicity [3–6], and recommendations for the validation of immunoassays have been described in Shanker et al. [4]. A strategy for the validation of immunoassays will be outlined in this chapter. Readers are referred to Chapter 15, for a detailed description of the validation of assays for neutralizing antibodies, and to the white paper by Gupta et al. [7], for the validation of cell-based assays for the detection of neutralizing antibodies (NAbs).

Detection and Quantification of Antibodies to Biopharmaceuticals, First Edition. Edited by Michael G. Tovey.
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It is generally accepted that a tiered testing approach should be used for assessment of the immunogenicity of a biopharmaceutical [3–6]. In such an approach samples are first screened for the presence of ADAs using an immunoassay. Samples that test positive are then further characterized in a confirmatory assay and then may be further analyzed to determine potency and/or reassayed for the presence of NAbs. The extent of immunogenicity testing is determined case-by-case using a risk-assessment approach based upon the perceived risk to patient safety [3–6]. For example, using such an approach the development of NAbs to a biopharmaceutical that cross-reacts with a nonredundant essential endogenous protein would be considered to present a potentially high risk to patient safety. In contrast, the development of binding antibodies to a monoclonal antibody, without an endogenous counterpart, that did not adversely affect drug pharmacokinetics or pharmacodynamics would be considered to present a low risk to patient safety.

For the purposes of this chapter it is assumed that an immunoassay for binding antibodies (BAbs) to the biopharmaceutical in question has been developed and that assay conditions and reagents have been optimized. It is also assumed that sample stability, the minimum required dilution (MRD), and the concentration of drug needed to consistently decrease a high positive sample below the assay cut point have also been established.

The following validation parameters will be discussed: assay cut point; specificity cut point; sensitivity; system suitability controls; precision; acceptance criteria; recovery; drug interference; and assay robustness.

13.2. SCREENING ASSAY CUT POINT

The assay cut point is defined as the level of the response of the BAb assay at and above which a sample is defined as positive and below which the sample is considered to be negative. The assay cut point is established by testing at least 50 individual samples, preferably from untreated patients from the disease indication, on three different days using two operators and a balanced design—for example, using 3 plates per day, or 9 plates in total, per operator. If such samples are not available, then individual samples from healthy volunteers or animals can be used to establish an initial assay cut point. Once the first pretreatment samples from patients with the specific disease indication become available, then the mean values of the samples should be verified to determine whether they are in the same range as the samples from healthy volunteers that were used to establish the initial cut point. If significant differences are detected, then an in-study cut point should be established using pretreatment samples from the disease indication, once clinical studies have been initiated. For clinical studies it is important that the highest level of sensitivity is attained; consequently the assay cut point should be set such that a 5% false positive rate is obtained in order to minimize false negative results. In contrast, for preclinical studies, a high false positive rate is not necessary as

the principal use of preclinical immunogenicity data is to help interpret the findings of toxicity studies. Consequently the cut point can be calculated as the upper boundary of a one-sided 99.9% prediction interval. For both preclinical and clinical studies it may be preferable to use a nonparametric approach (99.9th or 95th percentile) that is independent of a normal distribution of individual samples. When employing this approach, however, a higher number of samples (approximately 100) should be used. Outliers can be removed using the "box-plot" method: samples showing a response above the 75th percentile plus 1.5 × [(75th percentile) – (25th percentile)] or below the 25th percentile minus 1.5 × [(75th percentile) – (25th percentile)] are removed before calculating the cut point (see Chapter 16 for further details).

To determine whether a fixed or a floating cut point should be used, a single factor analysis of variance (ANOVA) test should be used to determine whether the means of the cut point runs are significantly different or not. If the precision (percentage coefficient of variation, %CV) of each run is greater than 15%, then Levene's test should be used to determine whether the variance of the runs is significantly different. A fixed cut point can be used if the means and variances of the runs are not significantly different ($p = <0.05$). Alternatively, a floating cut point should be used if the means of the runs are significantly different but the variances are not (Fig. 13.1). If a floating cut point has to be employed, then the negative control is used to normalize the cut point (Fig. 13.2). Three independent aliquots of the negative control are tested on each of, for example, 9 plates used to establish the assay cut point, and the mean response of the resulting values is determined. A normalization factor can then be determined from the response at the assay cut point minus the mean response of the negative control. If both the means and variance of the assay runs are significantly different, a dynamic cut point should be used. That is, the

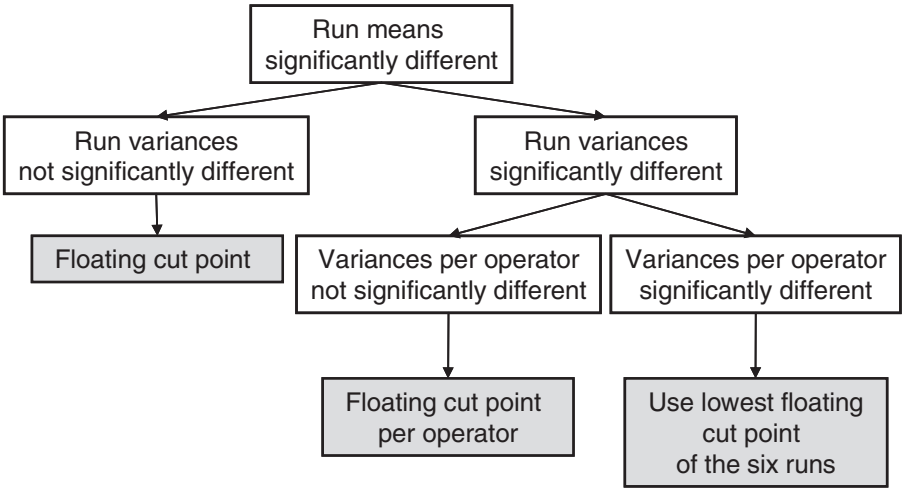

Fig. 13.1 Determination of the cut-point if run means are significantly different.

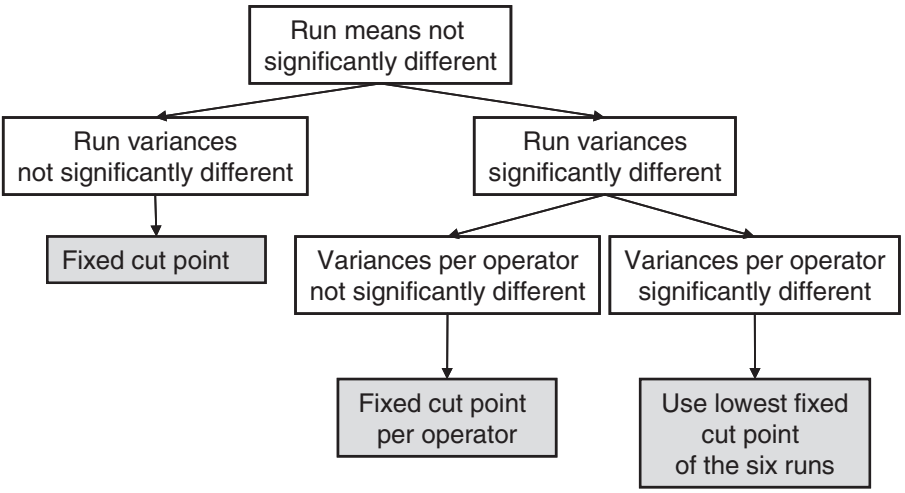
Run means not significantly different
Run variances not significantly different
Run variances significantly different
Fixed cut point
Variances per operator not significantly different
Variances per operator significantly different
Fixed cut point per operator
Use lowest fixed cut point of the six runs

Fig. 13.2 Determination of the cut-point if run means are not significantly different.

assay cut point should be determined on each plate using at least 20 individual samples. As this may not be feasible for a number of practical reasons, including limiting sample volume and the wells available per assay plate, alternatively an operator-specific cut point can be established provided that the assay variance per run does not differ significantly, in which case the lowest cut point of all six runs should be used. This may, however, lead to a higher false positive rate than an anticipated rate of 5%. This cut point should also be normalized using the negative control. A detailed discussion of the statistical validation of cut points is outlined in Chapter 16.

13.3. SPECIFICITY CUT POINT

As the screening cut point has been established such that a false positive rate of 5% is obtained, samples that test positive in the screening assay should be considered to be putative positive samples for the presence of BAb ADAs. Thus, in order to confirm that samples are indeed positive, samples are retested in the presence or absence of an excess of the specific drug. The specificity cut point is defined as the percentage inhibition at or above which a sample is considered as a confirmed positive sample. The specificity cut point is established during the validation of the screening cut point. Drug-naive samples from the population used to determine the screening cut point are spiked with an excess of drug and analyzed in an identical fashion, for example, 3 plates per day analyzed on three different days by two operators. The percentage inhibition for each sample is determined from the formula %(signal inhibition) = $100 \times \{1 - [(\text{study drug inhibited sample})/(\text{uninhibited sample})]\}$. Outliers can be removed using the box-plot method (see Section 13.2). The

specificity cut point will subsequently be computed as the upper boundary of a one-sided 99.9% prediction interval. It is preferable to use a nonparametric approach (99.9th percentile) as this is independent of a normal distribution.

13.4. SENSITIVITY—LOW POSITIVE CONTROL

The sensitivity, or lower limit of detection (LOD), of the BAb assay is determined by testing six independent dose–response calibration curves spanning the assay cut point. This is usually determined using serial dilutions of a strong positive control in the appropriate matrix (pooled sera or plasma) by two operators. Sensitivity is calculated from the *t*-distribution with 99% consistency of the back-calculated concentrations at the cut point. Thus, sensitivity equals the mean concentration at the cut point plus $t_{0.01.df} \times$ (standard deviation), where $t_{0.01}$ is the critical value determined from the *t*-distribution corresponding to a 1% false negative rate, and df is the degrees of freedom, which will depend upon the number of samples employed. To confirm the sensitivity, that is, the concentration corresponding to the LOD, approximately 10 individual samples, preferably from the disease indication, are spiked with a quantity of the positive control at slightly lower and slightly higher concentration than the theoretical LOD. The lowest concentration that tests positive in all individual samples, that is, at or above the assay cut point, will be defined as the sensitivity of the assay. This concentration will subsequently be used to establish the low positive control.

13.5. SYSTEM SUITABILITY CONTROLS, PRECISION, ACCEPTANCE CRITERIA

A low positive control at the LOD, a negative control (pooled serum), and a high positive control, situated in the linear range of the dose–response calibration curve close to the upper plateau, will be used as controls in the study. Thus, in order to define the precision and acceptance criteria for these controls they should be tested on three different days using three different plates per day, in parallel by two different operators. Each plate should also contain at least three different batches of the controls. It is not necessary to include the negative control in this determination, as it will have already been tested in a similar fashion in the screening cut point determination. To determine the precision and in-study acceptance criteria for the confirmatory assay, the low and high positive controls will also be tested after spiking with an excess of drug (the same amount as used for determination of the specificity cut point), and the percentage inhibition compared with the unspiked counterpart will be determined for each control.

Intrabatch and interbatch response and precision are also calculated for each control; that is, for example, for each operator nine intrabatches and one

interbatch for each control. The interbatch response and standard deviation will be used to define the acceptance criteria for each control for the screening assay with an objective of a 1% assay failure rate. Only an upper limit will be defined as the acceptance criterion for the negative control, to avoid an unusually high response impairing the assay sensitivity. Thus, the upper acceptance limit of the negative control equals the interbatch response plus $t_{0.01.df} \times$ (interbatch standard deviation). In contrast to the negative control upper and lower acceptance criteria will be defined for the low and high positive controls. The upper acceptance limit of the low and high positive control equals (interbatch response of the respective control) + [$t_{0.005,df} \times$ (interbatch standard deviation of the respective control)], whereas the lower acceptance limit of the low and high positive control equals (interbatch response of the respective control) − [$t_{0.005,df} \times$ (interbatch standard deviation of the respective control)]. The interbatch percentage inhibition and standard deviation will also be used to define the acceptance criteria for the low and high positive control for the confirmatory assay with an objective of a 1% assay failure rate. Thus, the upper (lower) acceptance limit of the confirmatory assay controls equals (interbatch inhibition) ± [$t_{0.005.df} \times$ (interbatch standard deviation)]. Once established, these acceptance criteria will also be used to assess the validity of batches within the validation study.

13.6. RECOVERY

Assessment of recovery in a BAb assay is based upon the determination of whether components in the assay matrix impair the binding of BAbs to the drug, thereby affecting assay results. To determine recovery from the target matrix samples, both low and high positive controls will be added to the assay buffer, target matrix (pooled sera), and at least 10 individual samples, preferably from the target indication, and tested at the MRD. The results obtained in assay buffer are then compared with those obtained in target matrix, and the percentage recovery is calculated. This procedure is carried out in parallel by two operators and, in addition to establishing assay recovery, will also confirm assay sensitivity and matrix interference and therefore will confirm the selected MRD. The percentage recovery has to fulfill a target acceptance criterion that has been defined during development, for example, ±30%.

13.7. DRUG INTERFERENCE

The principal interfering agent in a BAb assay is usually the drug itself. Interference will be observed when samples contain an excess of drug, due to competition for drug-specific antibodies between the drug present in the

sample and that immobilized on the surface of the assay plate, thereby resulting in false negative results.

To determine the drug tolerance of the BAb assay, both low and high positive controls will be spiked with increasing concentrations of drug until their response in the BAb assay falls below the assay cut point. The highest concentration of drug that still allows a positive response is defined as the drug tolerance level for the low and high positive controls, respectively. Due to differences in affinity and/or avidity the drug tolerance level thus determined may not reflect the true drug tolerance levels in study samples.

13.8. ROBUSTNESS

Robustness is an indication of the reliability of an assay, assessed by the capacity of the assay to remain unaffected by small deliberate variations in assay parameters. The objective of a robustness determination is to determine the consistency of assay results when subjected to relevant, real-life changes under routine laboratory conditions. The parameters to be analyzed for robustness are based upon the time- and/or temperature-sensitive steps observed during assay development. In addition, different batches of critical reagents should also be tested. The critical controls—negative control, low positive control, and high positive control—are used to assess the magnitude of changes in parameters such as temperature and/or incubation time that do not influence the validity of the BAb assay by determining whether the controls still fulfill their predefined acceptance criteria.

13.9. CONCLUSIONS

Assessment of immunogenicity is an important part of the safety evaluation for biopharmaceuticals. Consequently, it is critical to establish a validated assay that can detect clinically relevant events during the entire course of the clinical development of a biopharmaceutical, including postmarketing studies. A strategy and key performance criteria for the standardization and validation of immunoassays, based upon a tiered testing approach, have been outlined in his chapter. In such an approach samples are first screened for the presence of ADAs using a validated immunoassay, and samples that test positive are then further tested in a validated confirmatory assay. Additional analysis, which may include potency determinations, including titrations, and/or assays for the presence of neutralizing antibodies, are determined on a case-by-case basis using a risk-assessment approach based upon the perceived risk to patient safety [3–6]. The strategy outlined in the chapter is designed to ensure an efficient assessment of immunogenicity with optimal safety evaluation and an efficient use of resources.

REFERENCES

1. Neumann, T.A., Foote, M. (2000). Megakaryocyte growth and development factor (MGDF): an Mpl ligand and cytokine that regulates thrombopoiesis. *Cytokines Cell Mol Ther*, *6*, 47–56.
2. Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J.J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., Mayeux, P. (2002). Pure red-cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *N Engl J Med*, *346*, 469–475.
3. Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004). Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Meth*, *289*, 1–16.
4. Shankar, G., Devanarayan, V., Amaravadi, L., et al. (2008). Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*, *48* (5), 1267–1281.
5. Koren, E., Smith, H.W., Shores, E., Shankar, G., Finco-Kent, D., Rup, B., Barrett, Y.C., Devanarayan, V., Gorovits, B., Gupta, S., Parish, T., Quarmby, V., Moxness, M., Swanson, S.J., Taniguchi, G., Zuckerman, L.A., Stebbins, C.C., Mire-Sluis, A. (2008). Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products. *J Immunol Methods*, *333* (1–2), 1–9.
6. Ponce, R., Abad, L., Amaravadi, L., et al. (2009). Immunogenicity of biologically-derived therapeutics : assessment and interpretation of non-clinical safety studies. *Regul Toxicol Pharmacol*, *54*, 164–182.
7. Gupta, S., Indelicato, S.R., Jethwa, V., Kawabata, T., Kelley, M., Mire-Sluis, A.R., Richards, S.M., Rup, B., Shores, E., Swanson, S.J., Wakshull, E. (2007). Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. *J Immunol Methods*, *321*, 1–18.

INDEX

Acid dissociation, cell-based assays 327; *see also under* Soluble immune complexes
Adverse reactions 9, 114, 356, 357
  allergic reactions 289, 351, 355, 356, 359
  anaphylactic 8, 16, 19, 38, 51, 212, 214–216, 225, 227, 245
  anaphylactoid *see* anaphylactic
  Arthus reaction 97
  autoimmune 4, 16, 38, 40, 52, 71, 75, 243, 245, 289, 321, 394
  hypersensitivity 20, 25, 28, 175, 211, 212, 215, 216, 222, 225, 344, 351, 394
  infusion reactions 51, 214, 392
Affinity capture elution (ACE) 327
Aggregates *see under* Product-related factors
American Association of Pharmaceutical Scientists (AAPS) 259
Animal models 24, 41, 245
  Gottingen minipig 357
  transgenic for human proteins 42, 67, 331–341
Antibody, monoclonal 39, 41, 58, 321
Anti-drug antibodies
  anti-idiotype 71, 72, 89, 114, 115, 145
  anti-allotypic 89, 145
  anti-polyethylene glycol (PEG) 6, 20, 23, 26
  binding 47, 127, 135, 186, 236
  human anti-chimeric (HACA) 23, 74, 89
  human anti-mouse (HAMA) 74, 89
  IgA 23, 25, 89, 355, 356, 358, 359
  IgE 8, 19, 23, 25, 49, 211–227, 346, 355, 356, 359–361
  IgG 23, 25, 89, 114, 206, 207, 217, 218, 222, 225, 355
  IgG1 207
  IgG2 207
  IgG3 207
  IgG4 91, 207, 225, 323
  IgM 5, 23, 25, 89, 114, 203, 206, 207, 359
  low affinity 5, 23, 44, 106, 107, 114, 197, 323

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neutralizing 38, 74, 127, 135, 139, 141, 145, 186, 206, 243–267, 306, 309–317, 324–325, 358
neutralizing, cross-reactivity with endogenous protein 52, 243
pre-existing 19, 49, 89, 225, 299, 352
xenogenic 358
Antibody-dependent cellular cytoxicity (ADCC) 386, 25, 253
Asthma 215, 352, 355
Arthus reaction *see under* Adverse reactions
Assay characteristics
coefficient of variation 188, 189, 237
cross-reactivity 243
drug-ligand concentration 256
drug tolerance 240–241, 359, 373–374
EC_{50} 165, 189, 256, 263, 307, 333, 336
ED_{50} 48
endpoint 253–254
IC_{50} 112
lower limit of detection (LOD) 239
lower limit of quantification (LLOQ) 375, 379
minimum required dilution (MRD) 236, 240, 253, 260, 257, 290
precision 262, 304
robustness 263
sensitivity 261, 378
signal-to-noise 250, 252, 256, 290
specificity 261
upper limit of quantification (ULOQ) 377
variation, analytical 292
variation, biological 292
Z'-factor 290
Assay components
biotin-conjugated reagents 178–184, 187, 190
cell line 248–252
control, high positive 305, 306
control, low positive 239, 303–304, 306
control, negative 298–300, 306
controls 47, 49, 112, 186, 254–255, 260, 298–300, 302, 303, 306, 384, 385, 395
division-arrested frozen cells 252, 385, 390
matrix 49, 372
ruthenium-conjugated reagents 178–185, 187, 190, 326
standards 46, 264–265
Assay format 247–248
balanced design 290, 306
heterogeneous 323
homogeneous 323
optimal plate layout 305
Assay optimization 256–259
Assay standardization 264–265, 338
Assay titer 304
Assay type
cell-based 45, 105, 243–267, 324, 376, 384
competitive ligand-binding 45, 106, 175–192, 247, 326, 336, 384
confirmatory 26, 42, 44–45, 103–117, 176, 290, 292, 300–302
constant antibody 139, 271, 273, 277–279, 387
constant antigen 137, 271, 273, 275–277, 387
cytopathic effect (CPE) 70, 158, 168–170, 253, 264, 334, 337
dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) 43
drug quantification based neutralization (DQ-NAb) 371–381
electrochemiluminescent (ECL) 43, 68, 69, 107, 149, 193, 195, 196, 324, 326
enzyme immunoassay (EIA) 92, 95, 193, 194
enzyme-linked immunospot (ELISPOT) 43, 332
ELISA *see* Enzyme-linked immunosorbent assay
fluorescence resonance energy transfer (FRET) 43
G-protein-coupled receptor (GPCR) 250, 252
immunoassay 235–242, 376
kinase receptor activation (KIRA) 250, 253
multiplex PCR 162, 169
myxovirus protein A (MxA) 122–127, 137, 158, 162, 164, 165, 167, 170, 264, 335–336, 388

neutralization 6, 42, 45–46, 48, 49, 69, 105, 124, 126, 134, 146–149, 176, 269–286, 290, 327, 306–307, 309
one-step 383–398
radioallergosorbent test (RAST) 218, 219, 360
radioimmuno (RIA) 86, 92, 95, 334
radioimmunoprecipitation (RIPA) 68, 69, 107, 177, 193–196, 324, 334
real time reverse transcriptase polymerase chain reaction (RT-PCR) 123, 137, 157–174, 335, 336, 337
reporter gene 136, 139, 250, 253, 325, 383, 385–395
screening 26, 42, 43–44, 104–110, 176, 290–301
surface plasmon resonance (SPR) 5, 43, 44, 50, 68, 69, 107, 108, 177, 193–209, 218, 323, 359
TaqMan *see* real time reverse transcriptase polymerase chain reaction
Assay validation 289
cell-based assay 235, 259–264
immunoassay 235–242
SPR 196, 199–200

B-cell epitope determinations
in vitro 332
in vivo 332
in silico 332
Biacore *see* Surface plasmon resonance
Biomarker 122, 124, 126, 127
Biosimilar 37, 41, 51
Bone morphogenic proteins (BMP) 17

Cell lines
A 549 138, 141, 162, 163, 167, 168
Jurkat 149
L929 146, 147
U937 389
WEHI 164 146, 147, 190
Chronic myelogenous leukemia (CML) 23
Chronic pancreatitis (CP) 245, 354, 355
Clinical studies 44
phase I 48, 246
phase II 246
phase III 48, 246
Committee for Medical Products for Human Use (CHMP) 39, 42
Complement-dependent cytotoxicity (CDC) 253
Condyloma acuminata 23
Crohn's disease 23, 40, 83, 86, 88, 92, 93
Cut-point determinations 47–48, 236, 237, 238, 260, 372, 289–308
Bartlett's test 298
confirmatory 104, 108–113, 300–302
correction factor 295, 300
dynamic 296
fixed 295
fixed vs. floating 237, 301–302
flow chart 291, 293–294, 301
identification of outliers 293, 296–298
Levene's test 295, 296, 298
median absolute deviation (MAD) 297
nonparametric approach 295
outliers box-plot 296–297
screening 238, 291–296, 297
Shapiro-Wilk test 293
skewness coefficient 293
validation 302–303, 306–307
Cystic fibrosis (CF) 344, 348, 354, 355, 360, 361

Dilutional linearity 309–317
Dilution-response curves
four-parameter logistic curves 310
measurement bias 310
truncation bias 311, 314
Drug interference *see* Drug tolerance
Drug tolerance 258–259
cell-based assays 246, 261, 325
ECL assays 326
ELISA 323

Enzyme-linked immunosorbent assay (ELISA) 4, 43–44, 69, 85, 86, 89, 108, 122, 123, 158, 178, 193–196, 357, 359, 388
bridging 91, 107, 115, 323
drug tolerance 323

Enzyme-linked immunosorbent assay (ELISA) (*cont'd*)
epitope masking 323
indirect 106
MxA 136, 141, 167–169
screening assay 68
transgenic animals 331–341
EMEA *see* European Medicines Agency (EMA)
Enzyme replacement therapy 6, 7, 70, 74
European Medicines Agency (EMA) 37–56, 39, 141, 158, 246, 247, 280
Epitope masking, ECL assays 324
Epoetin *see* Erythropoietin
Eprex *see* Erythropoietin
Erythropoietin (EPO) 4–5, 16, 29, 38, 40, 52, 71, 75, 176, 243, 257–258, 321, 384, 385, 394

Fabry's disease 16, 338
Factor VIII 7, 16, 22, 29, 38, 175
Factor IX 7
Four-parameter logistic dilution-response curves 310

Gaucher's disease 16, 22, 29
Generally regarded as safe (GRAS) 347, 352
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *see* Assay type, RT-PCR
Good Laboratory Practices (GLP) 195
Good Manufacturing Practices (cGMP) 385
Granulocyte colony stimulating factor (G-CSF) 133, 324
Granulocyte-macrophage colony stimulating factor (GM-CSF) 19, 41, 45, 48, 133, 321, 176

Hairy cell leukemia 23
Hanganutziu-Deicher (HD) antigen 64
Hemophilia A 22, 29, 38
Hemophilia B 7
Hodgkin's lymphoma 41
Human growth hormone (hGH) 17, 18, 216
Human leukocyte antigen (HLA) 22, 63
Human serum albumin (HSA) 21, 71, 75; *see also* Product-related factors, formulation
Humira *see* Monoclonal antibodies, adalimumab

Identification of outliers 293, 296–298
Immune tolerance 289, 332
breakdown 17, 18, 63
ImmunoCAP 213, 218–220, 222, 227, 360
Immunogenicity prediction 19, 42, 245
Immunoglobulin
IgA 349, 350, 351
IgE 64, 69, 71, 351, 353
IgG 69, 349, 351
IgG4 323
IgM 62, 69
Immunosupressive therapy *see* Patient-related factors, concomitant treatment
Innovator product 37, 41, 51
In study acceptance criteria 305
Insulin 16, 17, 18, 19, 23, 24, 28, 42, 70, 175
Interferon 175, 384
alpha (IFNα) 19, 21, 23, 28, 29, 42, 58, 120–126, 133, 137, 175, 272, 280, 281, 321, 387, 388, 395
beta (IFNβ) 19, 22, 23, 24, 28, 29, 38, 42, 45, 58, 74, 120, 121, 122, 123, 133–137, 139, 141–143, 157, 158, 162, 164–170, 248, 254, 264, 272, 278, 280, 281, 321, 335, 384, 387–392, 394, 395
gamma (IFNγ) 58, 122, 272, 280, 281, 332
epsilon (IFNε) 387
kappa (IFNκ) 387
lambda (IFNλ) 272, 281
Interferon sensitive responsive element (ISRE) 122, 389
Interleukin -6 (IL-6) 279, 280, 281, 385
Internal ribosomal entry site (IRES) 390, 394

International Conference on Harmonization (ICH) S6 regulatory guidelines 245, 259
Interstudy acceptance criteria 305
Intravenous immunoglobulin (IVIG) 18
Investigational new drug (IND) application 24, 28, 197

Kaposi's sarcoma 23
Kawade methodology 139, 142, 166, 269–286, 336, 387, 393

Lysomal enzyme products *see* Enzyme replacement therapy

Major histocompatibility complex (MHC) antigens
  class I 20
  class II 19, 20, 63
Matrix effects 45, 189, 252, 257, 359, 376, 379, 384
Matrix interference *see* Matrix effects
Megakaryocyte growth and development factor (MGDF) 4–5, 16, 23, 29, 38, 176, 243, 245, 394
Meso Scale Discovery (MSD) 107, 149, 184, 187, 193, 326
Methotrexate (MTX) 93, 94
Minimum required dilution *see under* Assay characteristics
Monoclonal antibodies 74, 248, 321
  adalimumab 5, 6, 27, 36, 9, 40, 59, 71, 72, 84–89, 143–146, 149, 92, 97, 214, 215, 326, 388, 392, 393, 394
  basiliximab 215
  certolizumab 40, 84, 85, 392
  cetuximab 19, 50, 59, 64, 212–214, 222, 225
  daclizumab 215
  golimumab 84, 86, 89, 144, 392–395
  infliximab 5, 23, 58, 83–89, 92, 95, 97, 143–145, 214, 225, 227, 392, 393
  muromonab 216
  natalizumab 5, 16, 45, 119, 216
  OKT3 22
  omalizumb 215, 225
  rituximab 41, 58, 325, 326
Multiple sclerosis 22, 23, 28, 38, 120, 122, 123, 142, 280, 388
  relapsing-remitting (RRMS) 134, 135, 139, 157, 170, 388, 391, 392
Myxovirus protein A (MxA) *see under* Assay type

OKT3 *see under* Monoclonal antibodies
Optimal plate layout 305
Oral replacement therapy 343–367
Oral tolerance 349, 351
Orthogonality 107, 108
Outliers 293, 296–298
  biological 296
  analytical 296

Pancreatic exocrine insufficiency (PEI) 343–346, 348–354, 356, 358
Patient-related factors 17, 22–23, 38, 343, 343, 354
  age 41, 66
  concomitant treatment 20, 41, 66, 93, 94, 143–145, 392
  genetic background 6, 65
  immune status 65
  route of administration 24
Pharmacodynamic (PD) markers 6, 16, 42, 51, 114, 121, 122, 124, 263, 289, 357, 358, 388
Pharmacokinetics 6, 16, 42, 50, 51, 86, 97, 114, 175, 289, 357–359, 384
Pharmacopeia, U.S. 259
Pharmacovigilance *see* Post-market surveillance
Pompe's disease 7, 22
Post-market surveillance 7, 26, 60, 68, 70
Pre-clinical testing 66, 245, 331–341
  NAb assays 245
Product-related factors 17, 18–22,38, 60–65, 343, 354
  aggregates 18, 21, 26, 27, 64
  adjuvant effect 62, 65
  formulation 21, 65
  glycosylation 62, 388
  host cell proteins (HCP) 347, 361

Product-related factors (*cont'd*)
novel epitopes 18, 19
primary structure 62
process-related 62
storage conditions 21, 27, 65
Psoriasis 215
Pure red-cell aplasia 4–5, 16, 21, 29, 38, 40, 245

Radioallergosorbent test (RAST) *see under* Assay type
Receiver operating characteristics (ROC) curve analysis 46
Regulatory guidance
European 37–56, 106
Japanese 57–79
United States 15–35, 195
Reporter gene 325
Rheumatoid arthritis 5, 6, 19, 40, 83, 86, 92, 97, 143–146, 212, 214, 215, 394
Ribosomal RNA *see* Assay type, real time reverse transcriptase polymerase chain reaction
Risk-based testing strategy 3–12, 24, 43, 51, 104, 244, 298
Risk management 72–74, 223–226
Rituximab *see under* Monoclonal antibodies
Route of administration *see under* Patient-related factors

Sample, stability 262
Shapiro-Wilk test 293
Skewness coefficient 293
Skin-prick test (SPT) 224, 353, 360
Soluble immune complexes 7
acid dissociation 49, 258, 327, 374
Standards Committee of the International Society for Interferon and Cytokine Research 272
Surface plasmon resonance (SPR) *see under* Assay type
Systemic lupus erythematosus (SLE) 41

T-cell epitope determinations
in vitro 19, 42, 67, 332
in vivo 19, 42, 67, 332
in silico 19, 42, 67, 332
Testing strategy
tiered approach 42, 104, 176, 236, 244, 246
Thrombopoietin (TPO) *see* Megakaryocyte growth and development factor
Titer
cut-point 304
evaluation 304
precision 304
TNF-alpha 146, 281, 385, 393
TNF-alpha antagonists 146–149, 392–395
adalimumab *see under* Monoclonal antibodies
etanercept 84, 85, 88, 144, 145, 148, 215, 392, 393, 394
infliximab *see under* Monoclonal antibodies
Tolerance
immune 17–18, 388
oral 349, 351
Transforming growth factor beta (TGF-β) 41
Tysabri *see* Monoclonal antibodies, natalizumab

Units
International Unit (IU) 275
laboratory unit (LU) 274, 275, 387
tenfold reduction units (TRU) 140, 166, 269, 271, 272, 276–283, 387, 392
Untreated positive rate (UTPR) 110, 111, 113
U.S. Food and Drug Administration (FDA) 15, 16, 24, 25, 26, 27, 28, 29, 144, 215, 259

White papers 39, 105, 106, 235, 245, 259, 290, 306
World Health Organization (WHO)
guidelines 142, 158, 166, 271, 279, 336
International Standards 137, 167, 146, 254, 272–273, 275, 385